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### **REMARKS**

This paper is being filed in response to the Office Action dated November 20, 2003 that was issued in connection with the above-identified patent application. Applicants enclose herewith an Petition for Extension of Time pursuant to 37 C.F.R. §1.136(a) and the fee required under 37 C.F.R. §1.17(a)(2). Applicants respectfully request reconsideration of the instant application in view of the amendments and remarks presented herein.

Claims 14-33 are pending in the instant application. Claims 14 and 21-30 have been amended. These amendments are fully supported by the specification as originally filed, for example, at page 1, line 22 to page 2, line 1 and page 3, lines 2-8 and, therefore, do not constitute new matter. New claim 34 has been added. New claim 34 is supported by the application as originally filed, for example, at page 4, lines 3-13 and Examples 1-3. Claims 32-33 have been withdrawn as allegedly directed to a non-elected invention. Upon entry of the instant amendment, claims 14-31 and 34 will be pending. The status of claims 32-33 will turn on the Examiner's decision regarding Applicants request for rejoinder (below).

As a preliminary matter, Applicants thank the Examiner, Dr. Gerald Ewoldt, and the Examiner's supervisor, Dr. Christina Chan, for granting Applicant's request for a telephonic interview with Applicant's Attorney, Dr. Rochelle K. Seide, and Applicant's Agent, Dr. Guy F. Birkenmeier on March 3, 2004. Applicant's greatly appreciate the Patent and Trademark Office's customer friendly approach to inventors generally and to the instant application in particular.

### **INTERVIEW SUMMARY AND COMMENTS**

During the March 3, 2004 interview Applicants, through their representatives, discussed four issues with the Examiners. First, Applicants observed that the instant application

and claims are directed to methods for producing **mature** cells and invited the Office's attention to the Summary of the Invention at page 3, lines 7-8, which states "**mature** dendritic Langerhans type cells" (emphasis added).

Second, Applicants explained that the term "Langerhans-type" is an adjective that modifies "dendritic cell" in both the original claims, which recited "dendritic Langerhans-type cells," and the claims as amended on July 23, 2003, which recited "Langerhans-type dendritic cells." This meaning, Applicants asserted, would be clear to one of ordinary skill in the art in view of the Background of the invention (page 1, lines 11-27), which describes the well-known developmental progression of dendritic cells from either blood monocytes or blood stem cells via Langerhans cells. Applicants observed that both terms have been rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite and, therefore, invited suggestions from the Examiners. In response, Examiner Ewoldt suggested that "dendritic Langerhans cells" would overcome the outstanding rejection. Applicants, therefore, have amended the pending claims in accordance with the Examiner's suggestion.

Next, Applicants noted that the outstanding Office Action included comments regarding the histochemical markers CD80 and CD83 and inquired whether the Examiners believed that the amended language, *i.e.* "dendritic Langerhans cells," would raise any new concerns regarding these markers. Applicants pointed out that the first document listed on the Information Disclosure Statement filed July 23, 2003 indicates that a single antibody raised to CD83 is capable of recognizing both Langerhans cells (LCs) and dendritic cells (DCs). Applicants have enclosed an additional copy of this document for the Examiner's convenience. During the telephonic interview, Applicants asserted that this document demonstrates, as Applicants contended in the Amendment filed July 23, 2003, that the art does not recognize a

bright line of distinction between immature and mature cells, but rather considers the totality of the morphology and histology. The Examiners did not immediately identify any problems, but asked Applicants to present these arguments in written form for further consideration.

Applicants have done so herein (see below).

Lastly, in response to the new matter rejection in the outstanding Office Action, Applicants proposed amending claim 14 to recite “mammalian platelets” and requested feedback. The Office indicated that Applicants should formally present the amendment to the Examiner for consideration. Accordingly, Applicants have amended claim 14 herein.

## **RESPONSE TO OUTSTANDING OFFICE ACTION**

### **Request for Rejoinder**

Claims 32-33 have been withdrawn as allegedly drawn to a non-elected invention. The Office Action states that the invention under consideration is a “method for producing Langerhans (immature) dendritic cells” while claims 32-33 are directed to a method for producing mature DCs. The Office Action alleges that these methods are patentably distinct and that the originally presented invention has been constructively elected.

As discussed during the interview, the present invention and claims are not directed to immature cells. Rather, as disclosed in the Summary of the Invention, original claim 1 (paragraph b) and instant claim 14 (paragraph b), the present invention relates to methods of producing mature DCs. Applicants, therefore, respectfully request rejoinder of claims 32-33.

### **The Specification Fully Supports the Pending Claims**

Claims 24-31 have been rejected under 35 U.S.C. § 112, first paragraph as allegedly drawn to subject matter that was not sufficiently described in the specification to

enable one of ordinary skill in the art to make and use the claimed invention. The Office Action alleges that the specification fails to sufficiently demonstrate that the method of the instant claims would result in a “Langerhans cell, i.e., an immature DC as set forth by Steinman.” The Office Action additionally alleges that the specification does not provide a specific definition of either “dendritic Langerhans type cell” or “Langerhans-type dendritic cell.” Thus, the term “Langerhans cell” has been construed to have its usual meaning.

As Applicants indicated in the interview, the term “Langerhans-type” is an adjective that modifies “dendritic cell” such that it is mature DCs that are the subject of the instant invention. Applicants explained that the term “Langerhans-type” was intended to denote lineage from Langerhans precursors. Applicants have asserted that this interpretation would be clear to one of ordinary skill in the art in view of the Background of the invention provided on page 1, lines 11-27 of the specification. Applicants further assert that this meaning would have been clear to one of ordinary skill in the art in view of a review article published in *Nature* by Banchereau et al. (hereinafter “Banchereau”)(Document 8, IDS filed July 23, 2003), a courtesy copy of which is enclosed herewith. Applicants respectfully invite the Examiner’s attention to the fourth full paragraph of the left column on page 249, wherein Banchereau states that “[t]here seem to be several pathways to generate DCs.” Banchereau goes on to explain that CD34+ cells contain progenitors for two discrete populations of DCs, namely “the epidermal LCs and dermal or interstitial type DCs.” See Banchereau, page 249, paragraph spanning left and right columns. Thus, Banchereau uses the terms “epidermal LC” and “interstitial type DC” to refer to **mature** DCs. Applicants assert, therefore, that the terms “dendritic Langerhans type cell,” “Langerhans-type dendritic cell,” and “dendritic Langerhans cell” are equivalent to each other and would have all been clear to one of ordinary skill in the art at the time the invention was made.

In view of the Background of the invention and Banchereau together with Applicants amendment according to the Examiner's suggestion, Applicants respectfully request withdrawal of this rejection.

The Claims Relate to Morphologically and Histochemically Mature DCs

The Office Action dated April 23, 2003 states that the specification's description of the cells produced by methods of the invention as having low expression of CD80 and CD83 is not indicative of mature DCs.

Applicants traverse this assessment and assert that the art does not recognize an iron-clad distinction between immature and mature cells, but rather considers the totality of the morphological and histological indicators. Applicants respectfully invite the Examiner's attention to Satthaporn et al. (Document 4, IDS filed July 23, 2003) wherein the authors state that "DCs posses [*sic*] a heterogeneous haemopoietic lineage in that subsets from different tissues have been shown to posses [*sic*] a different morphology, phenotype and function." Satthaporn, abstract. Indeed, DCs possess a heterogeneous haemopoeitic lineage that, together with the culture conditions, influences the morphology and immunological reactivity of the resulting cells. *See e.g.* Schoppet M et al. (Document 2, filed July 23, 2003). Applicants respectfully invite the Examiner's attention to Palucka et al. which shows that cell culture conditions may directly influence the expression of CD1a and CD83. *See* Palucka KA et al., paragraph bridging pages 4590-4592 (Document 11, filed July 23, 2003). Applicants respectfully invite the Examiner's attention to the Pathology Outlines web page submitted with the IDS filed July 23, 2003, which indicates that both LCs and DCs may stain positively for CD83. Applicants also respectfully invite the Examiner's attention to Banchereau at Figure 2 on page 245, wherein the figure legend states: "Many monoclonals to antigens that are not rigorously DC-specific, nor

well understood, are still useful in identifying DCs. These are CD83, an immunoglobulin superfamily member....” Therefore, notwithstanding the low level of CD80 and CD83, Applicants contend that one of ordinary skill in the art would recognize that the cells produced by the methods of the invention are mature DCs.

The Claims Are Free of New Matter

Claims 14-22, and 27-29 have been rejected under 35 U.S.C. §112, first paragraph as allegedly being drawn to new matter in reciting "a medium containing platelets." The Office Action has alleged that the specification does not convey to one skilled in the art that applicants had possession of a method of producing Langerhans cells of any species using platelets from any species.

Applicants traverse this rejection and assert that the claims, as amended herein, are fully supported by the application as filed. Claims 14 and 28 have been amended to recite “a medium containing mammalian platelets.” Support for this language may be found in the specification as originally filed at, for example, page 2, lines 21-26 and Examples 1-6. Therefore, Applicants respectfully request withdrawal of this rejection.

Notwithstanding the foregoing argument, Applicants traverse this rejection as it applies to claims 23 and 27 and as it may be applied to claims 30 and 34. Claim 23 recites “the peripheral blood monocytes or the bone marrow cells with platelets of the same species.” Support for this language may be found in the specification as originally filed at, for example, page 2, lines 23-26 and Example 3. Claim 27 relates to a method in which mouse bone marrow cells are cultivated in the presence of rat platelets. Support for this claim may be found in the application as originally filed, for example, at page 4, lines 14-21. Claim 30 relates to a method in which the dendritic cell precursors and platelets may be independently selected from the group

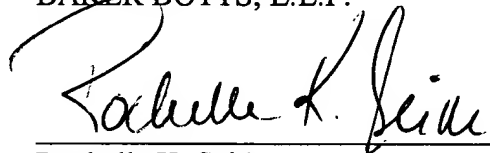
consisting of mouse and rat. Support for this claim may be found in the application as originally filed, for example, at page 4, lines 14-21. Claim 34 relates to a method in which the dendritic cell precursors and platelets are both human. Support for this claims may be found in the application as originally filed, for example, at page 4, lines 3-13 and Examples 1-3. Therefore, since claims 23, 27, 30, and 34 are free of new matter, Applicants respectfully request withdrawal of this rejection at least as it applies to these claims and claims dependent thereon.

In light of the foregoing amendments and remarks, Applicants believe that the instant application is in condition for allowance. Accordingly, Applicants respectfully solicit prompt favorable action on the instant application.

Applicants enclose herewith the fee required pursuant to 37 C.F.R. §§ 1.16(c) and 1.17(a)(1). While Applicants do not believe that any additional fee is required with this submission, the Commissioner is hereby authorized to charge any fees required with this submission not enclosed herewith to Deposit Account No. 02-4377. Two copies of this page are enclosed.

Respectfully submitted,

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March 15, 2004

Enclosures



# Dendritic cells and the control of immunity

Jacques Banchereau & Ralph M. Steinman

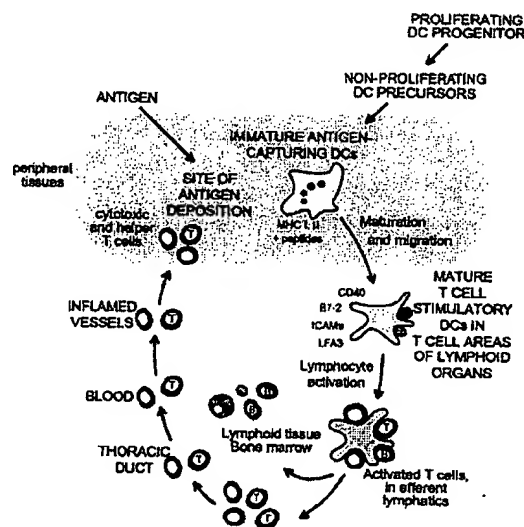
**B and T lymphocytes are the mediators of immunity, but their function is under the control of dendritic cells. Dendritic cells in the periphery capture and process antigens, express lymphocyte co-stimulatory molecules, migrate to lymphoid organs and secrete cytokines to initiate immune responses. They not only activate lymphocytes, they also tolerate T cells to antigens that are innate to the body (self-antigens), thereby minimizing autoimmune reactions. Once a neglected cell type, dendritic cells can now be readily obtained in sufficient quantities to allow molecular and cell biological analysis. With knowledge comes the realization that these cells are a powerful tool for manipulating the immune system.**

Immunology has long been focused on antigens and lymphocytes, but the mere presence of these two parties does not always lead to immunity. A third party, the dendritic cell (DC) system of antigen-presenting cells (APCs), is the initiator and modulator of the immune response. First visualized as Langerhans cells (LCs) in the skin in 1868, the characterization of DCs began only 25 years ago. It was known that 'accessory' cells were necessary to generate a primary antibody response in culture, but it was only once DCs were identified and purified from contaminating lymphocytes and macrophages that their distinct function as APCs became apparent (Box 1).

DCs are efficient stimulators of B and T lymphocytes. B cells, the precursors of antibody-secreting cells, can directly recognize native antigen through their B-cell receptors. T lymphocytes, however, need the antigen to be processed and presented to them by an APC. The T-cell antigen receptors (TCRs) recognize fragments of antigens bound to molecules of the major histocompatibility complex (MHC) on the surface of an APC. The peptide-binding proteins are of two types, MHC class I and MHC class II, which stimulate cytotoxic T cells (CTLs) and helper T cells respectively. Intracellular antigens, cut into peptides in the cytosol of the APC, bind to MHC class I molecules and are recognized by CTLs, which, once activated, can directly kill a target cell. Extracellular antigens that have entered the endocytic pathway of the APC are processed there and generally presented by MHC class II molecules to T-helper cells, which, when turned on, have profound immune-regulatory effects.

Some *raison d'être* for a specialized DC system are now clear (Fig. 1). The initiation of T-cell immunity is rather demanding. Initially, peptides from infected cells located anywhere in the body must be found and recognized by T cells that circulate in the blood stream. The amounts of specific antigen-MHC complexes on tumours and infected cells are typically small (one hundred or less per cell), and must be recognized by rare T-cell clones (usually at

a frequency of 1/100,000 or less) through a TCR that has a low affinity ( $1 \mu\text{M}$  or less). Moreover, infected cells and tumours frequently lack the co-stimulatory molecules that drive clonal expansion of the T cell, the production of cytokines, and development into killer cells. DCs provide a means of solving these challenges. Located in most tissues, DCs capture and process antigens, and display large amounts of MHC-peptide complexes at their surface (Fig. 1). They upregulate their co-stimulatory molecules and migrate to lymphoid organs, the spleen and the



**Figure 1** Afferent and efferent limbs of immunity that resolve several demands of antigen presentation *in vivo* (see text). Antigens are captured by DCs in peripheral tissues and processed to form MHC-peptide complexes. These immature DCs derive successively from proliferating progenitors and non-proliferating precursors, the latter not being fully committed to form DCs. As a consequence of antigen deposition and inflammation, DCs begin to mature, expressing molecules that will lead to binding and stimulation of T cells in the T-cell areas of lymphoid tissues. If the antigen has also been bound by B cells, then both B and T cells can cluster with DCs, as shown. After activation, T (blue) and B (orange) blasts leave the T-cell area. B blasts move to the lining of the intestine, the bone marrow, and other parts of the lymphoid tissue, such as the medulla of lymph node, with some becoming antibody-secreting plasma cells. T blasts leave the blood at the original site of antigen deposition, recognizing changes in the inflamed blood vessels and responding vigorously to cells that are presenting antigen. This limits the T-cell response to the site of microbial infection.

## Box 1 Dendritic cells and the control of immunity

- Sentinel *in vivo* *in situ* distribution to optimize antigen capture and migration into lymphoid organs to optimize clonal selection of rare B, T and CD8+ cells
- Initiators of immune responses: stimulation of quiescent naïve and memory B and T lymphocytes
- Potency in stimulating cells: capacity of small numbers of DCs and low levels of antigen to induce strong T cell responses
- Inducers of tolerance: deletion of self-reactive lymphocytes and variety of immature T cells

## review article

lymph nodes, where they liaise with and activate antigen-specific T cells (Fig. 1, right). All of these DC activities can be induced by infectious agents and inflammatory products, so that DCs are mobile sentinels that bring antigens to T cells and express co-stimulators for the induction of immunity.

Not only should the immune system attack that which is foreign or aberrant, it should also leave alone that which is neither to avoid autoimmunity. And again, DCs play a vital part. Normally, mature T cells do not respond to self-peptides that are presented to them: during their development only those T cells that have no, or low, affinity towards the peptide antigens present in the thymus are allowed to mature and to enter the circulation. But not all self-antigens are represented in the thymus. What prevents T cells from being activated by for example brain- or pancreas-derived self-peptides, an event that could ultimately result in the development of multiple sclerosis or diabetes? We shall discuss some evidence that DCs can tolerize the T-cell repertoire to self-antigens, both in the thymus and in the periphery.

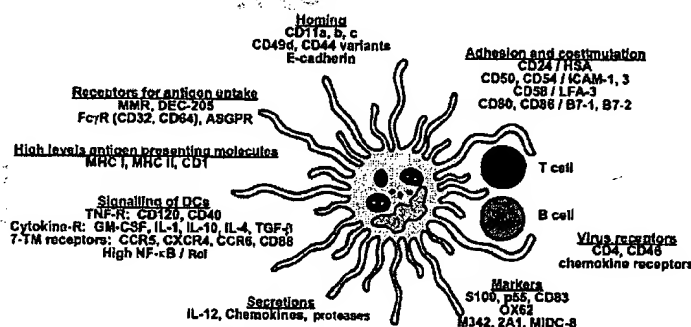
Until recently, the paucity of DCs and the lack of specific markers had impeded research. Both obstacles are being overcome, and sufficient DCs can now readily be prepared from progenitors<sup>2-10</sup>: bone-marrow cultures stimulated with cytokines, and mouse cell lines cultured in GM-CSF (granulocyte-macrophage colony stimulating factor) and interleukin (IL)-4, are both tricks of the trade used to obtain reasonable numbers of DCs. DCs derived from human blood monocytes that have been nurtured in GM-CSF and IL-4, followed by maturation in a monocyte-conditioned medium, are the most potent APCs known<sup>8,9</sup>. These DCs have many features

of primary DCs, including the expression of molecules that enhance antigen capture and selective receptors that guide DCs to and from several sites in the body (Fig. 2). New DC products are being rapidly identified by screening of complementary DNAs<sup>11-13</sup> that will provide more markers for identifying, characterizing and targeting DCs. We shall first review the features of mature DCs and their capacity to stimulate T cells, then discuss the immature antigen-capturing DCs, focusing on their migration to the lymphoid tissues and their development from progenitors; we shall consider their role in B-cell responses and the induction of tolerance and will finish by discussing their role in clinical immunology and pointing out the next challenges.

### Features of mature dendritic cells

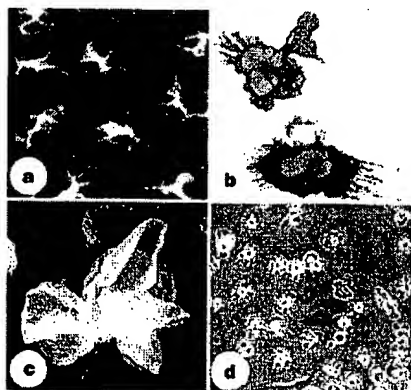
No other blood cell exhibits the shape and motility that give rise to the term 'dendritic' cell. *In situ*, as in the skin, airways and lymphoid organs, DCs are stellate (Fig. 3a). When isolated and spun onto slides, DCs display many fine dendrites (Fig. 3b). When looked at with an electron microscope, the processes are long (>10 µm) and thin, either spiny or sheet-like (Fig. 3c). When alive and viewed by phase-contrast microscopy, DCs extend large, delicate processes or veils in many directions from the cell body (Fig. 3d). These bend, retract, and re-extend in a non-polarized fashion for a day or more. Actin cables are scarce<sup>14</sup>. The shape and motility of DCs fit their functions, which are to capture antigens and select antigen-specific T cells.

Terminally differentiated or mature DCs can readily prime T cells (Box 1). Once activated by DCs, these T cells can complete the



**Figure 2** Some features of DCs, including DCs expanded *ex vivo* from precursors. Many monoclonals to antigens that are not rigorously DC-specific, nor well understood, are still useful in identifying DCs. These are CD83, an immunoglobulin-superfamily member; p55, a presumptive actin-binding protein; S100b,

a calcium-binding cytosolic protein; DEC-205, a multilectin in DCs and many epithelia; antibodies called M342, 2A1 and MDC-8 which recognize antigens in intracellular granules of mouse DCs; and OX62 on rat DCs and some T cells.



**Figure 3** The unusual shapes of DCs. a, DCs in a sheet of epidermis (MHC II stain); b, in cytopins stained for surface MHC II; c, by scanning electron microscopy; d, in the live state by phase-contrast microscopy.

immune response by interacting with other cells, such as B cells for antibody formation, macrophages for cytokine release, and targets for lysis. Immature DCs, on the other hand, are less potent initiators of immunity but specialize in capturing and processing antigens to form MHC peptide complexes. Thus, two key functions of DCs segregate in time: they first handle antigens and then, as mature DCs a day or more later, stimulate T cells.

*In vitro* or *in vivo*, only few DCs are necessary to provoke a strong T-cell response. *In vitro*, DCs can induce a so-called mixed leukocyte reaction (MLR), a model for graft rejection. Leukocytes from one individual, the potential transplant donor, are mixed with T cells from the responder or graft recipient. If donor and recipient are mismatched at the MHC, the T cells begin to proliferate, release cytokines and become CTLs. Normally, the MLR is carried out with equal numbers of stimulators and responders, but only one DC is necessary to turn on 100–3,000 T cells. These early observations introduced the notion that there are cells, DCs, specialized to initiate immunity. Now it is clear that DCs prime T cells not only to mismatched MHC, but to a range of foreign proteins, from superantigens, the microbial proteins that bind directly to MHC molecules without prior processing<sup>15</sup>, to the larger world of more standard proteins that do require processing, including those from infectious agents<sup>16,17</sup> and tumours<sup>18–21</sup>. *In vivo*, immunity develops in lymphoid organs, where the DC–T-cell interaction can be seen for all major classes of T-cell ligands<sup>22–24</sup>. DCs form clusters with antigen-specific T cells, creating a microenvironment in which immunity can develop<sup>22–24</sup>.

So far, no one has been able to identify a single specific molecule to explain the efficacy of DCs in T-cell binding and activation, and the special effects of DCs seem solely to relate to quantitative aspects and their regulation. For example, MHC products and MHC-peptide complexes<sup>25</sup> are 10–100 times higher on DCs than on other APCs like B cells and monocytes. Mature DCs resist the suppressive effects of IL-10, but synthesize high levels of IL-12 (refs 26–28) that enhance both innate (natural killer cells) and acquired (B and T cells) immunity. DCs also express many accessory molecules that interact with receptors on T cells<sup>29,30</sup> to enhance adhesion and signalling (co-stimulation), for example LFA-3/CD58, ICAM-1/CD54, B7-2/CD86. All these properties (MHC expression, secretion of IL-12 and the expression of co-stimulatory molecules) are upregulated within a day of exposure to many stresses and dangers, including microbial products.

Depending on the conditions, DCs can stimulate the outgrowth and activation of a variety of T cells, which affect the immune

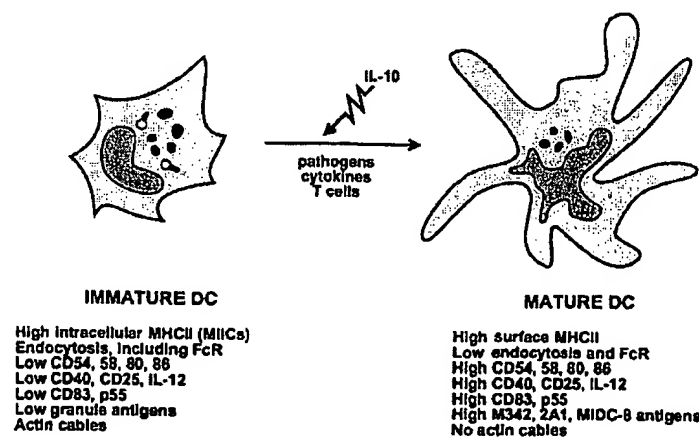
response differently. They can persuade CTLs, which express the accessory molecule CD8 and hence interact with MHC class I bearing cells, to proliferate vigorously, which is unusual for CD8<sup>+</sup> T cells<sup>31,32</sup>. CD4-expressing T-helper cells, on the other hand, scrutinize cells that express MHC class II molecules. In the presence of mature DCs and of the IL-12 they produce<sup>26–28</sup>, these T cells turn into interferon- $\gamma$  (IFN- $\gamma$ )-producing Th1 cells. IFN- $\gamma$  activates the antimicrobial activities of macrophages and, together with IL-12, it promotes the differentiation of T cells into killer cells. So the capacity of DCs to produce IL-12 and Th1 cells will lead to microbial resistance. With IL-4, however, DCs induce T cells to differentiate into Th2 cells which secrete IL-5 and IL-4. These cytokines activate eosinophils and help B cells to make the appropriate antibodies, respectively.

The communication between DCs and T cells seems to be a dialogue rather than a monologue in which the DCs respond to T cells as well. CD40 (ref. 33) and the newly described TRANCE/RANK receptor<sup>34,35</sup> on DCs are ligated by the TNF (tumour-necrosis factor) family of proteins expressed on activated and memory T cells: this leads to increased DC survival<sup>33,34</sup> and, in the case of CD40, upregulation of CD80 and CD86 (ref. 33), secretion of IL-12 (refs 26, 27) and release of chemokines such as IL-8, MIP-1 $\alpha$  and  $\beta$ <sup>33</sup>.

#### Immature antigen-capturing dendritic cells

In most tissues, DCs are present in a so-called 'immature' state, unable to stimulate T cells. Although these DCs lack the requisite accessory signals for T-cell activation, such as CD40, CD54 and CD86, they are extremely well equipped to capture antigens and—a key event in the induction of immunity—antigens are able to induce full maturation and mobilization of DCs.

The sentinel position of immature DCs stand out when the skin surface, or epidermis, is labelled for DC molecules (Fig. 3a). Humans have about 10<sup>9</sup> epidermal LCs, the immature dendritic cells of the skin that are located above the basal layer of proliferating keratinocytes. Freshly isolated LCs are weak T-cell stimulators, have few MHC- and accessory- molecules, but many antigen-capturing Fcy and Fce receptors. This phenotype changes dramatically within a day of culture (Fig. 4): the cells undergo extensive transformation, antigen-capturing devices disappear, and T-cell stimulatory functions increase. When a skin patch is explanted and the LCs are challenged with an antigen, the migration of mature DCs into the culture medium can be observed<sup>36</sup>. The situation is similar *in vivo*. When they encounter a powerful immunological stimulus, for example a contact allergen or a transplant, most of the LCs from



**Figure 4** Features that change during DC maturation. Immature DCs take up antigen in several ways: that is, phagocytosis, macropinocytosis or adsorptive pinocytosis. An example of a pathogenic molecule that will induce maturation is

lipopolysaccharide (LPS); TNF $\alpha$ , GM-CSF are examples of cytokines, and CD40L is an example of a T-cell ligand that binds CD40 on DCs. IL-10 can inhibit maturation.

## review article

the epidermis mature and move into dermal lymphatics in search of antigen-specific T cells. Small numbers of antigen-capturing DCs can also be isolated from blood, lung, spleen, heart, kidney, and the B- and T-cell areas of tonsils; these cells lack LC-specific markers (E-cadherin, Birbeck granules, Lag-1), but they also acquire accessory molecules within 1–2 days of culture, before any encounter with T cells. In summary, a pool of cells in our bodies seems to be committed to mature into potent antigen-presenting cells.

What is it that makes a DC such a good APC? Immature DCs have several features that allow them to capture antigen. First, they can take up particles and microbes by phagocytosis<sup>16,17,37,38</sup>. Second, they can form large pinocytotic vesicles in which extracellular fluid and solutes are sampled, a process called macropinocytosis<sup>7</sup>. And third, they express receptors that mediate adsorptive endocytosis, including C-type lectin receptors like the macrophage mannose receptor<sup>7</sup> and DEC-205 (ref. 39), as well as Fcγ and Fcε receptors<sup>6</sup>. Macropinocytosis and receptor-mediated antigen uptake make antigen presentation so efficient that picomolar and nanomolar concentrations of antigen suffice<sup>7</sup>, much less than the micromolar levels typically employed by other APCs. However, once the DC has captured an antigen, which also can provide a signal to mature, its skills to capture antigens rapidly decline, and the time has come to assemble antigen–MHC class II complexes.

The antigen enters the endocytic pathway of the cell. In macrophages most of the protein substrate is directed to the lysosomes, an organelle with only few MHC class II molecules, where the antigen is fully digested into amino acids. Not in DCs; the DC is able to produce large amounts of MHC class II–peptide complexes at a single brief stage of its life. Much of this success may be due to specialized, MHC class II-rich compartments (MIICs) that are abundant in immature DCs<sup>6,14,40,41</sup>. MIICs are late endosomal structures that contain the HLA-DM or H-2M products, which enhance and edit peptide binding to MHC class II molecules. During maturation of DCs, MIICs convert to non-lysosomal vesicles that discharge their MHC–peptide complexes to the surface<sup>41,42</sup> (Fig. 5). Immature DCs have been compared to idling motors in neutral gear, constantly degrading MHC class II molecules in their MIICs. As soon as an antigen instructs the DCs to move into gear, fragments of antigen are loaded onto class II molecules and these complexes are sent to the cell surface, where they remain stable for days<sup>41,42</sup>.

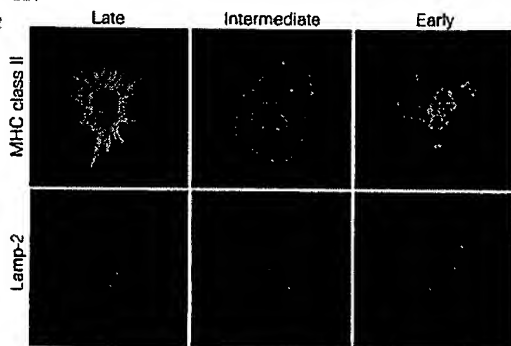
To generate cytotoxic killer cells, which have the capacity to eliminate infected cells and attack transplants and tumour cells, DCs

have to present antigenic peptides complexed to MHC class I molecules to CD8-expressing T cells. This is relatively straightforward if the DC is infected itself, with, for instance, influenza virus. The virus uses the cell's machinery to synthesize viral proteins, which are, like cellular proteins, degraded into peptides by the proteasome. A dedicated peptide transporter then translocates these peptides from the cytosol to the endoplasmic reticulum, where they bind to class I molecules. The peptide-loaded MHC class I complexes travel to the cell surface where they are displayed for scrutiny by T cells. It is less clear, however, how DCs could process and present antigens that have no access to the cytosol in an MHC class I-restricted manner (for instance transplant- and tumour-derived antigens, or antigens from viruses that cannot infect DCs). Nevertheless, they can probably present peptides from non-replicating microbes<sup>32</sup> and dying infected cells<sup>43</sup> by means of MHC class I as efficiently as if they were infected themselves. By processing dying cells, DCs may be able to 'cross-prime' or 'cross-tolerize' T cells to another cell's antigens or self-proteins, which could have major clinical implications, as we will discuss.

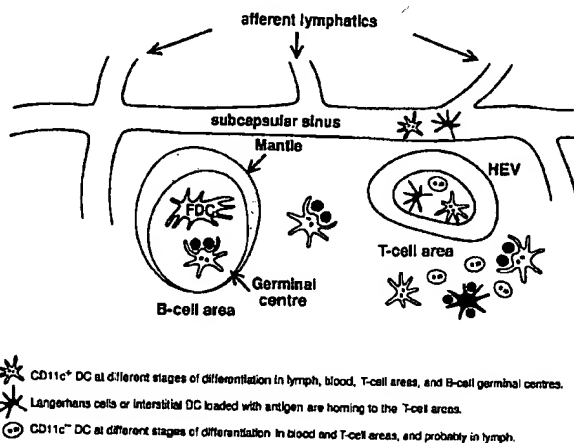
It is clear that maturation of DCs is crucial for the initiation of immunity. It can be influenced by a variety of factors, notably microbial and inflammatory products. Whole bacteria<sup>14</sup>, the microbial cell-wall component LPS<sup>7</sup>, and cytokines like IL-1, GM-CSF and TNF-α all stimulate DC maturation, whereas IL-10 blocks it<sup>44</sup>. Ceramide, which is induced by maturation signals, can shut down antigen capture by the DC<sup>45</sup>. Mature DCs express high levels of the NF-κB family of transcriptional control proteins (Rel A/p65, Rel B, Rel C, p50, p52)<sup>46</sup> which regulate the expression of many genes encoding immune and inflammatory proteins. Signalling through the TNF-receptor family, for example TNF-R (CD120a/b), CD40 and TRANCE/RANK, results in activation of NF-κB. Therefore, to induce the immune response through activation of DCs, a pathogen or antigen may have to engage the signal transduction pathways of the TNF-R family and TNF-R-associated factors (TRAFs).

### Migration of dendritic cells *in vivo*

Upon activation, DCs travel to the lymphoid tissues such as the spleen and lymph nodes. There, DCs may complete their



**Figure 5** Intracellular MHC II-bearing compartments in immature, maturing and mature DCs. MHC II is shown in green and lysosomal membrane glycoprotein (Lamp-2) (or H-2M) in red. Early or immature DCs have numerous MHC II-rich lysosomes (right); maturing DCs contain MHC II-positive, non-lysosomal vacuoles (green, middle panels) that probably bring large amounts of MHC II to the surface of late or mature DCs (left). Micrographs courtesy of S. Turley and I. Mellman.



**Figure 6** Distinct subsets of DCs in lymphoid organs, possibly derived from separate pathways of development (see text). DCs arrive through afferent lymphatics and HEVs and include cells that are on patrol, or have left peripheral tissues in response to a stimulus, or act as precursors to lymphoid DCs. All DCs may initially enter the T-cell regions to mature into what are called 'interdigitating' cells by electron microscopy. The activated B-cell follicles, with germinal centres, have two cell types: FDCs and CD11c<sup>+</sup> DCs, which present respectively native antigens to B cells and processed antigens to T-helper cells. HEV, high endothelial venule.

maturation<sup>47</sup>, attract T and B cells by releasing chemokines<sup>11</sup> and maintain the viability of recirculating T lymphocytes<sup>48</sup>.

Even in the absence of invading pathogens, a fraction of the DC population seems to move around. These cells are always present in afferent lymph that access the T-cell areas (Fig. 6), but not in efferent lymph, indicating that most of the migrating DCs die after their arrival in lymphoid tissues. In blood, there are two subsets of precursor DCs, one expressing and one lacking CD11c (ref. 49). Both subsets can enter lymphoid organs at high endothelial venules by virtue of CD49d  $\beta$ 1 integrins<sup>50</sup> possibly with different final destinations such as the B-cell (CD11c<sup>+</sup>) and T-cell (CD11c<sup>-</sup>) areas (Fig. 6). DCs along liver sinusoids move in hepatic lymphatics to coeliac lymph nodes<sup>24,51</sup>, and DCs from intestine migrate to mesenteric lymph nodes. The DCs that migrate in the steady state may replenish immature populations or may be on patrol to identify invaders, like other white blood cells.

Not every pathogen or antigen induces a strong T-cell response, but those that do can induce the mobilization and maturation of DCs. Inhaled viruses and bacteria mobilize DCs into airway epithelium, and this influx proceeds as rapidly as that of neutrophils<sup>52</sup>. An injection of LPS causes a massive egress of DCs from skin, heart, kidney and intestine<sup>53,54</sup>, as well as the maturation and movement of spleen DCs from the marginal zone into the T-cell areas<sup>55</sup>. Application of a fluorescent contact allergen to the skin induces DC migration, and a day later fluorescent DCs can be found in the draining nodes. Following transplantation of heart and skin, DCs crawl out of the graft and make their way to lymphoid tissues. When access is prevented by disconnecting the afferent lymphatics, no immune response to skin transplants and contact allergens develops.

How dendritic cells know where to go is largely unknown. LPS<sup>53</sup> stimulates a variety of cells to produce cytokines and chemokines, for example GM-CSF, TNF- $\alpha$ , IL-1, MIP-1 $\alpha$  and B. These products are known to modulate DC movement and maturation. Damage to cytokine-rich cells, such as keratinocytes or mast cells, induced by an antigen could result in the release of preformed mediators like GM-CSF, TNF- $\alpha$  and IL-4. The current focus, however, is on seven-transmembrane-spanning G-protein-coupled receptors, an ever-growing family of proteins that includes receptors for calcitonin-receptor-related peptide (which is found in nerve endings that abut on skin LCs<sup>56</sup>), C5a and chemokines<sup>13,57</sup>. These peptides and their receptors may mediate many of the steps required for the migration and targeting of DCs: egress from a tissue, directed movement or chemotaxis, and maintenance of viability.

### Dendritic cell development

There seem to be several pathways to generate DCs. We mentioned earlier that blood monocytes give rise to DCs when cultured with the appropriate cytokines<sup>6-9</sup>. The DC progenitors are also present in bone marrow: a small CD34<sup>+</sup> subset of haematopoietic progenitors gives rise to all blood cells and DCs. The DC progeny is likely to colonize most tissues *in vivo* as immature non-dividing cells. Several cytokines contribute to their growth and differentiation. Both c-Kit ligand and Flt-3 ligand are transmembrane proteins on stromal cells that bind to tyrosine-kinase receptors and sustain DC progenitors<sup>58</sup>; administration of Flt-3 ligand *in vivo* stimulates the outgrowth of functional DCs<sup>10</sup>. GM-CSF and IL-3 (refs 2, 59), products of activated T cells and other cells, also enhance DC differentiation, whereas macrophage (M)-CSF favours differentiation of the precursors into macrophages. TNF and CD40L<sup>33,60</sup> block the granulocyte-differentiation pathway and stimulate the final maturation of DCs.

Cells that express the marker CD34 contain progenitors for two discrete DC populations: the epidermal LCs and dermal or interstitial type DCs<sup>61,62</sup>. One functional difference is that only interstitial-type DCs directly stimulate naive B cells to make antibodies<sup>63</sup>. The LC progenitor expresses CLA (a ligand for E-selectin and a skin-homing molecule) and lacks CD14 (a marker that is abundant on

monocytes) and cannot form macrophages. In contrast, the dermal DC progenitors lack CLA, give rise to CD14-positive cells that resemble monocytes, and can form either macrophages in response to M-CSF or DCs in response to GM-CSF and TNF- $\alpha$ <sup>61,64</sup>. Mice deficient in TGF- $\beta$  mice have no epidermal LCs but maintain DCs in other sites<sup>65</sup>, which underscores the peculiarity of LCs.

A tentative but intriguing subset of DCs may be specialized in the induction of immune tolerance<sup>66</sup>. It is proposed that these DCs arise from a progenitor that also yields T cells<sup>67</sup>. IL-3, not GM-CSF, drives the development of these lymphoid DCs, which lack myeloid markers (markers shared by macrophages and granulocytes such as CD11b, CD13 and CD33)<sup>68</sup>. In humans, a distinct lymphoid DC precursor may have been identified; it expresses CD4, lacks CD11c and looks like an antibody-forming plasma cell but develops into a DC in the presence of IL-3 and CD40L<sup>69</sup>.

It is noteworthy that although mouse DC lines can be generated with the help of transforming viruses, there are no examples of spontaneous DC malignancy. In human Langerhans cell granulomatosis, originally called histiocytosis X, typical LCs and eosinophils accumulate. The cause of these granulomas is unknown, but they appear to be clonal in origin and create symptoms as a result of their position in critical sites rather than by overt malignant transformations.

### Dendritic cells and B lymphocytes

DCs, famous for their T-cell-stimulatory properties, are now known to have major effects on B-cell growth and immunoglobulin secretion. B cells and DCs are both APCs and both are essential for antibody responses but for entirely different reasons (reviewed in ref. 1; Box 2); DCs activate and expand T-helper cells, which in turn induce B-cell growth and antibody production. But during this *ménage à trois*, there is more direct DC-B-cell dialogue as well (Fig. 1).

Naive B cells respond uniquely to the interstitial, non-LC type of DC<sup>63,70</sup>, and by secretion of soluble factors<sup>70</sup>, including IL-12, DCs stimulate the production of antibodies directly and the proliferation of B cells that have been stimulated by CD40L on activated T cells. DCs also orchestrate immunoglobulin class-switching of T-cell-activated B cells: IL-10 and TGF- $\beta$  can induce secretion of IgA<sub>1</sub>, but expression of IgA<sub>2</sub> appears to be strictly dependent on a direct interaction between the B cell and the DC<sup>71</sup>. This indicates that DCs are in control of mucosal immunity, and, in fact, DCs can be found in mucosal lymphoid tissues beneath antigen-transporting M cells<sup>72-74</sup> and in T-cell areas.

Follicular dendritic cells, or FDCs, directly sustain the viability, growth and differentiation of activated B cells (Fig. 6). They also organize the primary B-cell follicles, as shown by the absence of FDCs and follicles in TNF- $\alpha$ -knockout mice. FDCs differ from ordinary DCs: they are not bone-marrow-derived<sup>75</sup>, they lack the leukocyte marker CD45, and they display a unique set of molecules at their surface (reviewed in ref. 76), including all known complement receptors (CD11b, a long isoform of CD21 (ref. 77) and CD35). With their receptors for complement and Fc, FDCs capture antibody-antigen complexes and display whole complexes, rather than processed antigens, at their surface for long periods. FDCs are abundantly present within antigen-stimulated B-cell areas, or germinal centres. There, proliferating B cells (centroblasts) undergo

### Box 2 Differences between dendritic cells and B cells as APCs

- DCs have higher levels of MHCII and accessory molecules
- DCs make large amounts of IL-12
- DCs can internalize antigens by means of Fc and mannose receptors; B cells have antigen-specific immunoglobulin receptors and inhibitory FcR

## review article

somatic mutation, after which they stop dividing (centrocytes) and wait to be triggered by an immune complex on FDCs. B cells that recognize an immune complex with high affinity process the antigen and present it as peptide-MHC complexes to antigen-specific T cells. The T-B-cell interaction ensures the survival of these high-affinity B cells, while the non-stimulated low-affinity B cells apoptose and are phagocytosed by tingible body macrophages.

It is now known that germinal centres contain a second type of dendritic cell, the CD11c<sup>+</sup> DC (Fig. 6). It also carries immune complexes, and is a much more powerful stimulator of T cells than the germinal centre B cells<sup>78</sup>. This may be the DC that brings the antigens to the germinal centre and displays processed antigens to memory T cells.

### Dendritic cells and T-cell tolerance

Most studies focus on the power of DCs to activate T cells, but before T cells encounter foreign antigens, the T-cell repertoire should be tolerized to self-antigens. This occurs in the thymus (central tolerance) by deletion of developing T cells, and in lymphoid organs (peripheral tolerance) probably by the induction of anergy or deletion of mature T cells. In both cases, the DC system that initiates immunity to foreign antigens also appears to tolerize T cells to self-antigens.

In the thymic medulla, DCs present self-antigens in the context of MHC molecules. Thymocytes that have too high an affinity for self-antigens are deleted (negative selection). If antigen-bearing DCs are directly injected into the developing thymus or fetal thymic organ cultures, reactive T cells are deleted. In the thymic cortex, macrophages digest large numbers of dying thymocytes that have failed to undergo positive selection. As these macrophages handle large amount of self-antigens, they seem ideally suited to delete auto-reactive T cells, yet they do not seem to do so: if MHC class II molecules are solely present on DCs in the medulla, negative selection ensues<sup>79</sup>. If, on the other hand, MHC class II molecules are only expressed by cortical epithelium, and not by DCs in medulla, the propensity to autoimmunity increases<sup>80</sup>, indicating that DCs in the medulla are responsible for the deletion of auto-reactive T cells. Also, *in vitro* it is the DC, and not the macrophage, that efficiently deletes auto-aggressive thymocytes<sup>81</sup>.

Recent studies point to an important role for DCs in the induction of peripheral tolerance as well. DCs can capture and present self-antigens that are exclusive to specialized tissues. For example, bone-marrow-derived APCs present peptides, which are derived from insulin-producing  $\beta$ -cells of the pancreas, to T cells in the draining lymph nodes<sup>82-84</sup>, tolerance ensues, probably as a result of T-cell anergy or deletion<sup>83,84</sup>. It has recently been shown that DCs present peptides from apoptotic cells<sup>85</sup>. Accordingly, DCs may be able to present many self-antigens, derived from the normal turnover of somatic cells, to T cells and thus induce tolerance to self-proteins that have no access to the thymus.

What determines whether a DC turns the immune system on or off? Lymphoid DCs are long-lived cells and express very high levels of MHC-self peptide complexes<sup>25</sup>, and maybe T cells become anergic or die in response to abundant and persistent antigens. Maybe distinct DCs are responsible for the different tasks: more resident lymphoid DCs induce tolerance to self, whereas migratory myeloid DCs, including LCs, are activated by foreign antigens in the periphery and move to lymphoid organs to initiate an immune response. Another possibility is that tolerance-inducing DCs are qualitatively different, perhaps expressing a death molecule like Fas ligand<sup>66</sup>.

### Dendritic cells in clinical immunology

Given their central role in controlling immunity, DCs are logical targets for many clinical situations that involve T cells: transplantation, allergy, autoimmune disease, resistance to infection and to tumours, immunodeficiency, and vaccines. In autoimmune diseases

such as psoriasis and rheumatoid arthritis, increased numbers and activation of DCs have been noted. DCs are important APCs in the lung, possibly contributing to allergy and asthma. In transplantation and contact allergy, DCs have been implicated in the induction of both immunity and tolerance. Here we concentrate on three areas where direct data on the role of DCs is available: infection with immunodeficiency viruses, resistance to tumours, and new approaches to vaccination.

Recent results paint a paradoxical picture in which DCs, instead of inducing host resistance, provide a safe haven for several viruses. Cells of the DC system may be hosting latent cytomegalovirus<sup>85</sup>, and Kaposi's virus (KSHV) may likewise be sheltered in patients with multiple myeloma<sup>86</sup>. For HIV-1 and measles, the consequences of DC infection are more overt: especially upon interacting with memory T cells and activated T cells, they sustain the production of many HIV-1, SIV and measles<sup>87-93</sup> particles. Measles turn DCs into multinucleated cells, or syncytia, and suppresses dendritic-cell and T-cell function<sup>91,93</sup>. HIV-1 and SIV also vigorously replicate in DC-derived syncytia *in vitro*<sup>90,94</sup>, but immunosuppression is not apparent at this time. Syncytia are not just a sign of viral toxicity, as is often assumed, but are also true virus factories<sup>90-94</sup>. *In vivo*, infected syncytia have been noted on the surfaces of mucosa-associated lymphoid tissues<sup>72,73</sup>. These so-called lymphoepithelia contain numerous memory B and T cells, as well as DCs that are chronically exposed to maturation stimuli from the environment. But, rather than battling with the infection, mature DCs assist in its spreading by transmitting HIV-1 and SIV to T cells<sup>87,88,90,94</sup>. FDCs in B-cell areas (Fig. 6) appear not to be infected with immunodeficiency virus, but they may play a dual role. By displaying virions complexed with antibody, they nevertheless can elicit resistance, especially B-cell memory, but at the same time they act as long-lived extracellular reservoirs of potentially infectious virus.

Many tumour components do not elicit an antigen-specific T-cell response in patients, which may be due to the absence of functional DCs in tumours. DCs that infiltrate colon and basal-cell skin cancers can lack CD80 and CD86 (ref. 95) and therefore have reduced T-cell stimulatory activity. Likewise, tumours may secrete factors, such as IL-10, TGF- $\beta$  and vascular endothelial growth factor, that reduce DC development and function. Be that as it may, the more DCs infiltrate the tumour, the better the prognosis.

The immune repertoire carries tumour-reactive T cells, especially CTLs, but there is little evidence that these T cells are being activated *in vivo*. However, when tumour antigens are applied to DCs *ex vivo* and these DCs are then reinfused, specific immunity ensues. In animals this strategy can lead to protection against tumours and even a reduction in the size of established tumours<sup>96-98</sup>, and at present similar studies are carried out in patients. Many vehicles for the delivery of tumour antigens to DCs are being considered: viral vectors, naked and plasmid DNA, RNA, liposomes with nucleic acid or protein, and tumour lysates, apoptotic cells and peptides. It is interesting that DCs appear to have a direct lytic potential on certain tumour targets as well<sup>99</sup>.

Vaccine design has yet to target the DC system. DCs can readily elicit helper and killer T cells, antibodies and IL-12, and can operate at mucosal surfaces where protection is needed early during many infections. In contrast, many existing vaccines and adjuvants are weak stimulators of CD8<sup>+</sup> T cells and Th1-type T cells. As some DCs appear to tone down the immune response, vaccines that target these DCs could also be used to induce tolerance, for example to allergens.

The classical approach to vaccination exploits attenuated forms of pathogens to elicit an immune response, and such attenuation is now more feasible using genetic manipulation and new vectors like avipox viruses. *In vitro*, DCs are the only cells that efficiently present inactivated virus<sup>32</sup>, and therefore the efficacy of the new generation of attenuated vaccines could be improved by specific targeting to DCs. The immune response can also be boosted by immunization



with DNA vaccines; even though the DNA is primarily expressed in weak APCs, like dermal and muscle cells, DNA vaccines can activate both CD4- and CD8-bearing cells. DCs isolated from vaccinated animals both express the vaccine DNA<sup>100,101</sup> and present the corresponding peptides to specific T cells<sup>101</sup>. The frequency of transfected DCs is low and greater efficiency should prove valuable.

### Upcoming challenges

We have only just started to appreciate the extraordinary life of dendritic cells and many questions remain unanswered. At the molecular level, there is a sizeable effort to screen expressed sequence tags from DCs to characterize their products. Regulatory molecules of the killer-inhibitor family<sup>102</sup>, of chemokines and chemokine receptors<sup>11,13,103</sup>, and of proteinases<sup>12</sup> have now been identified. This ongoing search should uncover new lymphocyte-binding and antigen-processing molecules, secretory products, and transcription factors that may help to explain the specialized features of DCs.

At the physiological level, many features of DCs need to be understood in more detail to allow successful manipulation of the immune system. What signals lead to maturation and migration of DCs, how do they know where to go, and how do they reach the lymphatics, T-cell areas, and sometimes B-cell areas? What are the roles of the different DC subsets, including their effects on B cells and tolerance? Have DCs a larger repertoire of secretory products than currently appreciated, and are some of these, like IL-12, immune modulators? And, what determines whether a DC turns the immune system on or off?

At the clinical level, the efficacy of DNA vaccines and vectors may crucially depend upon their capacity to transduce DCs. Vaccines in general should improve if they elicit mobilization and maturation of DCs. Emerging evidence points towards a role for DCs in tolerance, and it will therefore be imperative to pursue DCs more vigorously in allergy, transplantation and autoimmunity. On the other hand, many microbes such as HIV-1 and measles may take advantage of the special features of DCs. Widely used immunomodulators like steroids may block DC maturation<sup>97</sup>, and perhaps new drugs can be designed to modulate their functions. The results are awaited of clinical trials in which DCs are being used to elicit immunity to viral infections and tumours.

Although studies of pathogenesis have long focused on the core of immunology by identifying antigens and lymphocytes, all the recent evidence places a new emphasis on the role of DCs in the control of immunity.

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## CD83<sup>+</sup> Dendritic Cells in Inflammatory Infiltrates of Churg-Strauss Myocarditis

Michael Schöppet, MD; Sabine Pankuweit, PhD; Bernhard Maisch, MD

• Churg-Strauss syndrome (CSS) is characterized by a granulomatous vasculitis of multiple organs with the cardiovascular system being commonly affected. The initiation of the disease is not well understood, but immunologic phenomena are thought to contribute at least partially to the syndrome. We have studied endomyocardial biopsy specimens from a patient with CSS and eosinophilic myocarditis for characterization of infiltrating immune cells by immunofluorescence staining techniques and found a major population to be composed of CD83<sup>+</sup>CD14<sup>+</sup>CD19<sup>+</sup>CD56<sup>+</sup>HLA-DR<sup>+</sup> dendritic cells (DCs). Further phenotypic characterization of infiltrating CD83<sup>+</sup> cells in CSS myocarditis showed a surface profile reminiscent of immature DCs. In the same patient, the cytokine expression pattern of mitogen-stimulated peripheral blood mononuclear cells revealed a T<sub>H</sub>0 response as evidenced by multiplex polymerase chain reaction, and interleukin-5 levels were elevated in plasma analyzed by enzyme-linked immunosorbent assay. Thus, in this case of CSS myocarditis, we found DC-like cells in myocardial lesions, which may suggest that DCs are involved in the inflammatory process possibly triggered or sustained by an imbalance of DCs and their failure to confer tolerance to self-antigens. (*Arch Pathol Lab Med.* 2003;127:98-101)

Churg-Strauss syndrome (CSS), first described in 1951,<sup>1</sup> belongs to the group of small vessel vasculitides, such as Wegener granulomatosis and microscopic polyangiitis, that are associated with antineutrophil cytoplasmic antibodies (ANCA) in the sera of patients. Main target antigens recognized by ANCA are the myeloperoxidase in CSS and serine protease 3 in Wegener granulomatosis. Antibody titers may correlate with activity of the disease, although in CSS up to 50% of patients have no detectable circulating antibodies. On activation by ANCA, polymorphonuclear neutrophils become cytotoxic, especially for endothelial cells, and release proinflammatory cytokines, which attract monocytes and T cells. Myeloperoxidase and serine protease 3 antigen-specific T cells have been isolated from peripheral blood in ANCA-associated vasculitis patients, and T-cell persistence might contribute to the propensity of patients to relapse.<sup>2</sup> Cardiac involvement is an important cause of morbidity and mortality in CSS and

includes pericarditis, eosinophilic myocarditis, vasculitis, and obliterative or restrictive cardiomyopathy that may progress to endomyocardial fibrosis.<sup>3</sup>

Among antigen-presenting cells, the dendritic cell (DC) system functions to initiate and modulate specific immune responses. Bone marrow-derived DCs have been demonstrated to be the most potent activators of naive T-lymphocyte responses. Besides the capacity of DCs to activate T cells and to initiate immunity to foreign antigens, the DC system also seems capable of tolerizing T cells to self-antigens, and in autoimmune diseases such as rheumatoid arthritis or psoriasis, increased numbers and activation of DCs have been noted,<sup>4</sup> supporting the delicate balance among DCs, self-antigens, and T cells.

### REPORT OF A CASE

A 50-year-old woman met the definition for CSS proposed by the Chapel Hill Consensus Conference<sup>5</sup> and fulfilled the criteria of the American College of Rheumatology. After a symptom-free interval of 9 years with an initial presentation of asthma, polyneuropathy, pericardial effusion, and eosinophilia and after a regimen of azathioprine for 2 years with gradually reduced doses of steroids, she presented at the hospital with shortness of breath. Chest radiography revealed pulmonary congestion and cardiomegaly. On echocardiography, left ventricular ejection fraction was reduced to 30%, the left atrium and left ventricle were enlarged, and a small posterior pericardial effusion was seen. A complete blood cell count showed a normal white blood cell count, but a marked eosinophilia of 39%, and total immunoglobulin E (IgE) concentration was elevated. Heart catheterization revealed no stenotic lesions, and endomyocardial specimens were obtained by left ventricular endomyocardial biopsy after informed consent and on approval of the local ethics committee, showing eosinophilic tissue infiltration and microgranulomas. Combination therapy of steroids and azathioprine and heart failure treatment were initiated. Four months after onset of symptoms, the left ventricular ejection fraction was 45%, no pericardial effusion was detectable, and no signs of pulmonary congestion were seen on chest radiography. Eosinophilia normalized to 1%.

### PATHOLOGIC FINDINGS

Immunohistochemical studies were performed on snap frozen biopsy specimens, conserved in liquid nitrogen. Afterward, they were embedded in compound medium (Tissue-tek, Sakura Finetek, Torrance, Calif) and subsequently placed on poly-L-lysine-treated glass slides. Fixation was achieved by 100% ice-cold acetone, and specimens were blocked with 5% goat serum in phosphate-buffered saline. Incubation with a first monoclonal antibody and the first detection antibody, a fluorescein-conjugated, goat anti-mouse IgG Fab fragment (Dianova, Hamburg, Germany;

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Antibodies Used in the Study*				
Antigen	Clone	Species/Isotype	Major Specificities	Supplier
CD1a	HI149	Mouse, IgG1	Langerhans cells, cortical thymocytes, subsets of DCs	Pharmingen, San Diego, Calif
CD3	UCHT1	Mouse, IgG1	All T cells	Dako Corporation, Hamburg, Germany
CD4	MT310	Mouse, IgG1	T-cell subset, monocytes, macrophages	Dako
CD14	MØP9	Mouse, IgG2b	Monocytes, macrophages, granulocytes	Becton Dickinson, Heidelberg, Germany
CD19	4G7	Mouse, IgG1	B cells	Becton Dickinson
CD56	T199	Mouse, IgG1	NK cells, T-cell subset	Dako
CD80	L307.4	Mouse, IgG1	Activated B cells, T cells, monocytes	Pharmingen
CD83	HB15A	Mouse, IgG2b	Mature DCs	Immunotech-Coulter, Krefeld, Germany
CD86	IT2.2	Mouse, IgG2b	Germinal center cells, activated B cells, monocytes	Pharmingen
HLA-DR	L243	Mouse, IgG2a	Monocytes, macrophages, DCs, B cells	Becton Dickinson

\* DCs indicates dendritic cells; NK, natural killer.

dilution 1:100), followed. Afterward, staining with a second monoclonal antibody and detection by a rhodamine red-X-conjugated, goat anti-mouse IgG antibody (Dianova; dilution 1:100) completed staining of sections. The panel of antibodies used in this study are listed in the Table. Biopsy specimens were microscopically analyzed using a Leica TCS-NT confocal laser microscope (software package TCS-NT/SP 1.6.587). The infiltrates showed strong immunoreactivity for CD83 (Figure 1). Contrarily, myocardial infiltrates of 5 patients with origin of myocarditis distinct from CSS did not reveal substantial numbers of CD83<sup>+</sup> DCs (data not shown). A large number of CD83<sup>+</sup> cells exhibited single staining for this selective DC surface marker, and little coexpression of leukocyte markers, such as CD14 (macrophages/monocytes), CD19 (B lymphocytes), or CD56 (natural killer cells; Figure 1, d through f), was detected. Further surface staining revealed a DC phenotype reminiscent of immature DC (weak CD80 and CD86 expression, no CD1a expression; data not shown).

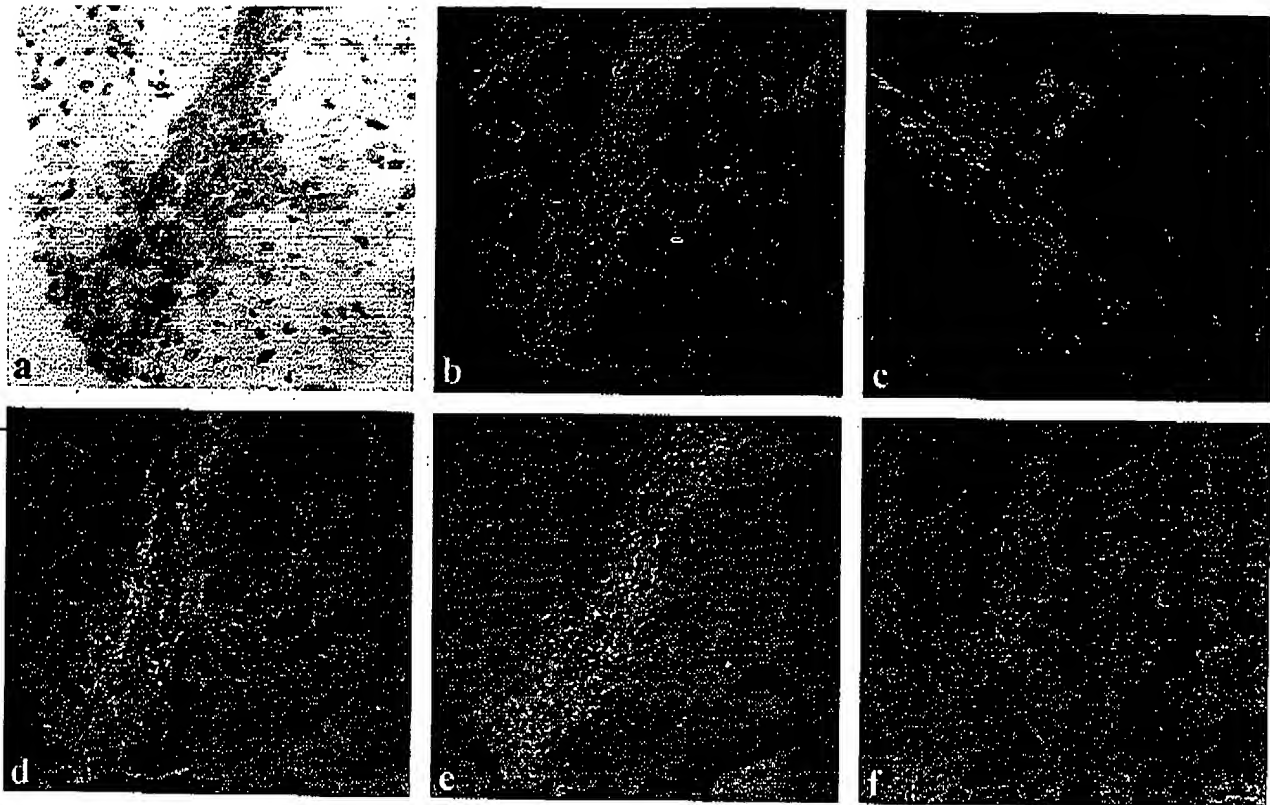
To obtain peripheral blood mononuclear cells (PBMCs), whole blood was subjected to a density gradient centrifugation, and isolated cells were stimulated by phytohemagglutinin-P (PHA-P; Sigma, Deisenhofen, Germany) at a concentration of 10 µg/mL for 12 hours in RPMI medium. The RNeasy Mini Kit (Quiagen, Hilden, Germany) was used for parallel generation of PBMC-derived DNA and RNA. DNA was digested by DNase (Promega, Mannheim, Germany), and whole extracts were amplified by an intron-spanning primer specific for NF-κB to verify complete digestion. RNA was used as a source for cDNA. Cytokine complementary DNA and glyceraldehyde-3-phosphate dehydrogenase for positive control were detected using the multiplex polymerase chain reaction system (Biosource, Nivelles, Belgium) according to the instructions of the manufacturer. Finally, samples were transferred to a 2% agarose gel containing 0.5 µg/mL of ethidium bromide. A T<sub>H</sub>0 cytokine profile after stimulation with the mitogen PHA-P could be demonstrated by this method. Before stimulation, only a faint band indicating interleukin (IL)-10 expression could be visualized, whereas after stimulation, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, and interferon-γ; expression with a dominant IL-2 signal was observed (Figure 2).

Various cytokines were investigated in plasma by use of the OptEIA enzyme-linked immunosorbent assay (Becton Dickinson, Heidelberg, Germany), following exactly the instructions of the manufacturer. Only IL-5 levels proved to be elevated, whereas none of the other cytokines, namely IL-2, IL-4, IL-6, interferon-γ, tumor necrosis factor α, or transforming growth factor β, could be detected (Figure 3).

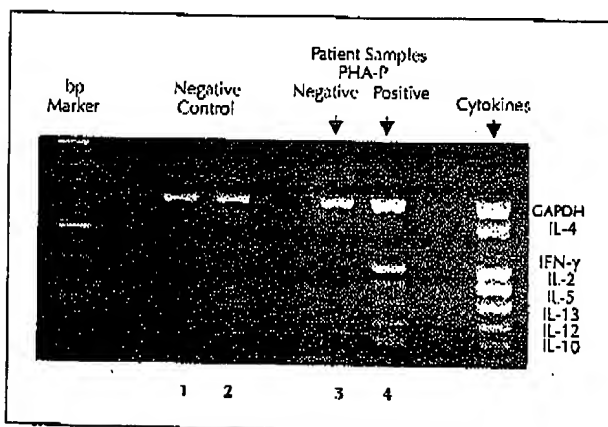
#### COMMENT

The origin of CSS is still under investigation. Some aspects of the pathogenesis of the disease were studied in the past, with focus on ANCA-triggered activation of neutrophils and subsequent damage to the endothelium.<sup>2</sup> In this study, we are describing the detection of CD83<sup>+</sup> DCs in inflammatory infiltrates of CSS myocarditis, in contrast to myocarditis infiltrates with an origin distinct from CSS, in which we were unable to find substantial CD83 immunoreactivity. CD83, a member of the immunoglobulin superfamily, has been proven to be a suitable and selective cell surface marker for mature DCs, which seems to be involved in regulation of immune responses by binding counterreceptors on monocytes and activated T cells.<sup>6</sup> Since we did not find costaining for CD1a and CD83 in our specimens, it is therefore conceivable that CD1a<sup>+</sup>/CD83<sup>+</sup> cells in CSS myocarditis represent an immature subset of DCs with intracellular expression of CD83 instead of mature DCs, which cannot be determined from immunohistochemical analysis. Double staining with lineage markers typical for macrophages/monocytes and B lymphocytes revealed a minor number of CD83<sup>+</sup> cells exhibiting coexpression. Further characterization of CD83<sup>+</sup> cells showed weak costaining for the costimulatory molecules CD80 and CD86, but brighter staining for major histocompatibility class II (HLA-DR) molecules. Because of the limitations of a report on a single patient, it is hard to predict whether DC-like cells are a common feature in all patients with CSS myocarditis. It would be interesting to compare lesions in different organ systems, such as kidney or lung, with our findings.

On investigating the cytokine expression pattern of PBMCs in the same patient after mitogen stimulation, we found a T<sub>H</sub>0 profile with a dominant IL-2 signal. Increas-

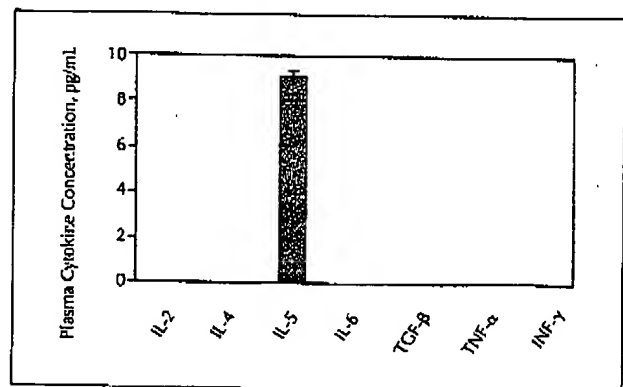


**Figure 1.** Hematoxylin-eosin staining of an endomyocardial biopsy specimen from a patient diagnosed as having Churg-Strauss syndrome myocarditis (a). High numbers of infiltrating cells can be detected (original magnification  $\times 400$ ). b, Overlay image of the same infiltrate stained by an immunofluorescence technique using monoclonal antibodies against CD83 (green fluorescence) and CD3 (red fluorescence). c, Overlay image of a second infiltrate of the same patient. Green fluorescence represents staining for CD83, whereas red fluorescence represents staining for CD4. Coexpression leads to yellow staining (see arrowheads). Coexpression of surface markers of macrophages/monocytes, B lymphocytes, or natural killer cells on CD83<sup>+</sup> cells of the first infiltrate were studied by double immunofluorescence staining and overlay technique. CD83 is represented by green fluorescence, whereas CD14 (d), CD19 (e), and CD56 (f) are represented by red fluorescence. Coexpression of these markers leads to yellow staining. Note that most of the infiltrating cells exhibit single green fluorescence staining (see arrowheads), and the concomitant CD56 (neural cell adhesion molecule) staining of myocytes (original magnification  $\times 630$  [b-f]).



**Figure 2.** Cytokine expression of peripheral blood mononuclear cells (PBMCs) in Churg-Strauss syndrome myocarditis. The PBMCs were isolated at the time of the endomyocardial biopsy without (3) and with (4) stimulation by phytohemagglutinin-P (PHA-P) as evidenced by multiplex polymerase chain reaction. A 100-bp marker (bp) and negative control [PBMC cytokine expression in a control patient; (1) unstimulated PBMCs, (2) PHA-P-stimulated PBMC] are indicated. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; and IFN- $\gamma$ , interferon- $\gamma$ .

ing evidence suggests that  $T_H1$  responses are involved in autoimmune diseases. Contrarily,  $T_H2$  cytokines are thought to exert anti-inflammatory functions by negatively regulating  $T_H1$  cell-mediated immunity, thus limiting the



**Figure 3.** Cytokines in Churg-Strauss syndrome myocarditis were measured in plasma by an enzyme-linked immunosorbent assay system in parallel to cytokine production of peripheral blood mononuclear cells. IL indicates interleukin; TGF- $\beta$ , transforming growth factor  $\beta$ ; and INF- $\gamma$ , interferon- $\gamma$ .

detrimental effects of the  $T_H1$  response.<sup>7</sup> Although few studies have focused on cytokine production in CSS, a recent study has also found a  $T_H0$  profile in T-cell lines from patients with CSS, but with predominance of IL-4 and IL-13 production.<sup>8</sup> In plasma, we found elevated IL-5 levels, which is in accordance with a previous report,<sup>9</sup> demonstrating elevated IL-5 levels in serum and bronchoalveolar fluid of patients diagnosed as having CSS.

Because T lymphocytes seem to be involved at least in sustaining the inflammatory process in systemic vasculitides, T cells depend on the presentation of antigenic peptides for proliferation and clonal expansion. Endothelial cells seem to be incapable of expressing, processing, or presenting ANCA antigens.<sup>2</sup> Of interest, even B-cell-deficient mice develop myocarditis after challenge with cardiac myosin,<sup>10</sup> suggesting that activation of pathogenic T cells is accomplished by DCs or macrophages. Increased numbers of DCs point to a disturbance in the balance between DCs and T cells. Because DCs are believed to confer self-tolerance to self-antigens, a missing propensity of the DC system to do so might trigger autoimmune disease (ie, systemic vasculitides). On activation by ANCAs, neutrophils are cytotoxic for endothelial cells, and cell damage in CSS might release intracellular antigens, which commonly are not freely available. Furthermore, disintegration of the endocardium or the endothelium of intracardial vessels might offer an entrance site for blood-derived DCs to the interstitial spaces of the heart, where antigen presentation to T cells leading to inflammatory cascades can commence. Besides deposition of immune complexes to perivascular spaces, this DC-dependent presentation of

self-antigens to T cells might represent another pathomechanism for tissue and cell injury in CSS. Repeated antigen presentation to circulating T cells during remission of the disease might lead to a relapse of symptoms. However, further work has to be performed for elucidation of the exact pathogenesis of CSS and systemic vasculitides in general with respect to the unique DC system.

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# Dendritic Cells as the Terminal Stage of Monocyte Differentiation<sup>1</sup>

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Monocytes (MO) cultured for  $\geq 5$  days with either macrophage-CSF (M-CSF) or granulocyte macrophage (GM)-CSF and IL-4 differentiated without concomitant proliferation into CD14<sup>+</sup> macrophages (M $\phi$ ) or CD1a<sup>+</sup> dendritic cells (DC), respectively. When adherent and nonadherent CD14<sup>high</sup> M $\phi$  from M-CSF cultures were separated and cultured further in cytokine-free medium or with GM-CSF/IL-4, most cells from both fractions that were exposed to GM-CSF/IL-4 acquired CD1a expression and DC morphology and function. Conversely, GM-CSF/IL-4 withdrawal at day 5 and additional culture of sorted CD1a<sup>+</sup> DC for 2 to 7 days in cytokine-free medium led to cells rapidly becoming adherent CD1a<sup>+</sup>CD14<sup>+</sup> M $\phi$ . Replacing GM-CSF/IL-4 with M-CSF hastened the conversion of DC to M $\phi$  without increasing cell numbers. CD1a<sup>+</sup>CD14<sup>+</sup>CD83<sup>+</sup> mature DC were induced by a  $\geq 2$ -day exposure to MO-conditioned medium, LPS, or TNF- $\alpha$ /IL-1 $\beta$ . Upon cytokine removal or culture with M-CSF, DC that had been pushed to maturation by conditioned medium or LPS remained stable or died in the new environment. TNF- $\alpha$ /IL-1 $\beta$ -driven DC displayed heterogeneous CD83 expression and could thus be sorted into CD83<sup>high</sup> and CD83<sup>low/-</sup> cells; in cytokine-free medium or in M-CSF, most CD83<sup>low/-</sup> cells converted to M $\phi$ , whereas most CD83<sup>high</sup> cells remained nonadherent CD1a<sup>+</sup>CD14<sup>+</sup> or died and thus appeared truly terminally differentiated. Hence, MO are precursors of M $\phi$  as well as of DC, with each cell type having the capability to convert into the other until late in the differentiation/maturation process. Accordingly, the cytokine environment and the presence of differentiation and/or other stimulatory signals may be the "final decision-making factors" determining whether these cells will acquire DC or M $\phi$  characteristics and function. *The Journal of Immunology*, 1998, 160: 4587–4595.

The efficient generation of adaptive Ag-specific immune responses depends upon early Ag uptake and subsequent presentation to T cells in lymphoid organs (1–3). Professional APCs such as dendritic cells (DC),<sup>2</sup> macrophages (M $\phi$ ), and B lymphocytes are able to ingest, process, and present Ag in the context of MHC molecules (2, 4). However, these cells have different immunologic functions; DC are the most potent APC and are able to induce primary responses both in vitro and in vivo (1, 4, 5). Some DC functions, particularly the induction of secondary immune responses, can be substituted for by M $\phi$  (4).

Several lines of evidence point to DC and M $\phi$  being offspring of the same CD34<sup>+</sup> hematopoietic progenitor cell (reviewed in Refs. 4 and 6). Culturing purified cord blood CD34<sup>+</sup> cells with granulocyte macrophage (GM)-CSF and TNF- $\alpha$  leads to the generation of CD1a<sup>+</sup> Langerhans cells and CD14<sup>+</sup> precursors of DC; the latter display dual differentiation potential, as they can become either CD1a<sup>+</sup> DC, when cultured with GM-CSF/TNF- $\alpha$  and/or upon the addition of IL-4, or M $\phi$ , when in the presence of macrophage-CSF (M-CSF) (7, 8). DC can also be generated at a later stage of CD14<sup>+</sup> cell development. Indeed, we and others have shown that monocyte (MO)-enriched PBMCs cultured with GM-CSF/IL-4 differentiate into nonadherent (NA) CD1a<sup>+</sup>CD14<sup>low/-</sup> cells with the morphologic and functional characteristics of DC (MO-derived DC (MDDC)); but, these cells are immature as defined by lack of CD83 expression (9–15). The generation of MDDC occurs without concomitant proliferation, suggesting true differentiation rather than selection of less mature precursor cells (13–15). Alternatively, culturing MO in M-CSF leads to the generation of CD14<sup>high</sup> adherent (ADH) M $\phi$  (11, 15). These data clearly show the double differentiation potential of MO and/or their immediate precursors. However, it has not yet been determined whether MDDC or M $\phi$  are endpoints of MO differentiation or whether MO, DC, and M $\phi$  are interconverting populations, the "final" phenotype of which depends on the prevailing cytokine environment. Therefore, we investigated whether MDDC and M $\phi$  can truly interconvert into one another and, if so, at which stages of their differentiation and maturation this occurs.

## Materials and Methods

### Media and reagents

Unless otherwise indicated, the culture medium used was composed of RPMI 1640, 1% L-glutamine, 1% penicillin/streptomycin (Life Technologies, Paisley, U.K.), 50  $\mu$ M 2-ME (Sigma, St. Louis, MO), and 10% FCS

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<sup>4</sup> Abbreviations used in this paper: DC, dendritic cells; M $\phi$ , macrophage; GM-CSF, granulocyte macrophage-CSF; M-CSF, macrophage-CSF; MO, monocyte; MDDC, monocyte-derived DC; CM, conditioned medium; PI, propidium iodide; MPO, myeloperoxidase; MFI, mean fluorescence intensity; NA, nonadherent; ADH, adherent; PE, phycoerythrin; SI, stimulation index.

(Dutscher, Brumath, France). The following human recombinant cytokines were used: GM-CSF (5 ng/ml; Schering-Plough, Kenilworth, NJ), IL-4 (250 U/ml), IL-1 $\beta$  (100 U/ml), TNF- $\alpha$  (100 U/ml) (all from Genzyme, Cambridge, MA), and M-CSF (50 U/ml; R&D Systems, Minneapolis, MN). Conditioned medium (CM) was produced by MO stimulation in IgG-coated plates as previously described (16, 17). LPS (*Escherichia coli* 0111:B:4, Sigma) was used at 100 ng/ml.

### Cell purification

As previously described (15), MO were obtained from Ficoll (Seromed, Biochrome, Berlin, Germany) separated PBMC of healthy volunteers, depleted of T and B lymphocytes using M-450 Pan B/CD19 and M-450 Pan T/CD2 Dynabeads (Dyna, Oslo, Norway) at a (1:1):1 bead:cell ratio. The recovered population routinely contained >80% CD14<sup>+</sup> cells.

T lymphocytes (>95% CD3<sup>+</sup>) were obtained by immunomagnetic bead depletion of PBMC using a mixture of CD19, CD14, CD56 (Becton Dickinson Immunocytometry Systems, San Jose, CA), CD16, CD13, and glycoporin A (Immunotech, Marseille, France) mAbs, and subsequent incubation with goat anti-mouse M-450 Dynabeads (Dyna) at a 20:1 bead:cell ratio. Alternatively, T cells were purified (>90% CD3<sup>+</sup>) by rosetting (18).

### Culture conditions

MDDC or M $\phi$  were generated by a 5- to 7-day culture of MO-enriched PBMC ( $2 \times 10^6$  cells/3 ml) in culture medium supplemented with GM-CSF/IL-4 or M-CSF, respectively (9, 15), that was conducted in Costar plates (Cambridge, MA) unless otherwise indicated. In some experiments, Teflon dishes (Saville, Minnetonka, MI) were used to culture otherwise ADH cells. Cultures were fed every 2 days by removing one-third of the supernatant and adding fresh medium (half of the culture volume) with full doses of cytokines.

To induce the maturation of MDDC, day 5 cells were collected, GM-CSF and IL-4 were washed away, and the cells were replated at  $1$  to  $2 \times 10^6$  cells/3 ml culture medium supplemented with maturation-inducing factors (16, 17) (see Results).

### Cytokine switch and separation of cell populations

Cytokine switch in GM-CSF/IL-4 cultures was performed as follows: cells were collected and, after a short centrifugation, supernatant was discarded; cells were replated and cultured further in cytokine-free medium or with M-CSF. MDDC were sorted according to CD1a and/or CD83 expression (routinely >95% purity after sort) with a FACStar<sup>Plus</sup> (Becton Dickinson) and used for additional studies. Alternatively, cells were separated by direct and/or indirect panning with plastic-immobilized primary or secondary Abs. Unlabeled cells and/or CD1a- (Ortho Diagnostic Systems, Raritan, NJ) or CD14- (LecM3, Becton Dickinson) labeled cells were layered on Ab-coated plates in PBS plus 2% FCS and then incubated for 1 h at 4°C. Next, supernatant was collected, plates were gently rinsed, and residual cells were cultured in fresh medium and new cytokines (i.e., M-CSF or GM-CSF/IL-4 were added to CD1a<sup>+</sup> or CD14<sup>+</sup> cells, respectively). Incubation of cells with CD1a mAb did not result in additional activation, as no modification of the intracellular Ca<sup>2+</sup> concentration was found (data not shown and (19)).

ADH and NA cells of M-CSF cultures were separated by the removal of NA cells that were transferred to new wells with new cytokines. Wells were rinsed thoroughly to collect all NA cells, and fresh medium with new cytokines was added on the remaining ADH cells. If necessary, ADH M $\phi$  were detached by incubation for 10 min at 37°C in 0.02% EDTA/PBS.

### Multiparameter flow cytometry analysis

Cell surface marker expression was evaluated by double or triple immunofluorescence staining with the following mAbs: CD2, CD3, CD14 (LecM3), CD20, CD80, and HLA-DR (all from Becton Dickinson); CD1a (Ortho Diagnostic); CD83 (Immunotech); and CD86 (PharMingen, San Diego, CA). After both a 45-min incubation at 4°C and washing, cells were either fixed in 1% paraformaldehyde/PBS or resuspended in PBS plus 0.5% propidium iodide (PI) to exclude dead cells from analysis.

The intracellular expression of CD1a, CD14, CD68, and myeloperoxidase (MPO; the latter two from Dakopatts, Glostrup, Denmark) was evaluated following cell membrane staining, PermeaFix (Ortho Diagnostic) treatment for both cell fixation and permeabilization (45 min at 20°C), and incubation (45 min at 20°C) with the relevant FITC-conjugated mAb at saturating concentrations. After final washing, cells were resuspended in 1% paraformaldehyde/PBS.

Analysis was performed with a FACScan or a FACScalibur (Becton Dickinson). Marker expression was evaluated as the percentage of positive cells among MDDC or M $\phi$  defined by forward scatter/side scatter charac-

teristics and/or CD1a or CD14 expression, respectively. The distinction between low and high expression was based on the evaluation of mean fluorescence intensity (MFI).

### Functional studies

The MLR was performed using culture medium supplemented with 10% heat-inactivated normal human AB serum. Allogeneic T cells ( $5 \times 10^4$ /well) were cultured for 5 to 6 days in 96-well culture microplates (Costar) as responder cells to  $0.5 \times 10^3$  to  $10 \times 10^3$  MDDC or M $\phi$ . [<sup>3</sup>H]dThd incorporation (specific activity = 1 Ci/mM) (Amersham, Little Chalfont, U.K.) was measured after 8- to 16-h pulses with 1  $\mu$ Ci/well. Results are shown either as mean cpm of triplicates or as stimulation indices (SI) calculated by dividing mean cpm in test wells by mean cpm in control wells without stimulating cells.

Dextran-FITC uptake (0.1–1 mg/ml; Sigma) was evaluated by flow cytometry following 15 to 30 min of incubation at 4°C and/or 37°C and extensive washing with cold PBS.

## Results

### M-CSF-driven M $\phi$ convert to MDDC in a GM-CSF/IL-4-dependent manner

We have previously shown that a fraction of M $\phi$  obtained by the culture of MO with GM-CSF or M-CSF can acquire CD1a expression and DC morphology upon delayed addition of IL-4 or replacement with GM-CSF/IL-4, respectively (15).

Here, we evaluated this M $\phi$  plasticity and the potential of M $\phi$  to convert into MDDC to a greater extent. To this end, we separated the ADH and NA fractions of M-CSF-driven M $\phi$ , which we recultured after switching cytokines. Table I summarizes the characteristics of M $\phi$  after 5 to 7 days of M-CSF culture in Costar wells. Upon 4 to 7 days of reculture in GM-CSF/IL-4, most NA cells of M-CSF cultures (initially  $95 \pm 5\%$  CD14<sup>high</sup>, membrane and intracellular CD1a<sup>+</sup>) down-regulated CD14 expression (only  $12 \pm 6\%$  remained CD14<sup>low</sup>) and  $45 \pm 25\%$  acquired de novo CD1a (Fig. 1). The conversion potential of ADH cells from the same cultures was lower, with only  $27 \pm 26\%$  of cells becoming NA and CD1a<sup>+</sup>CD14<sup>+</sup> upon reculture in GM-CSF/IL-4 (Fig. 1). In both fractions, CD14 expression remained stable when cultures were pursued with M-CSF or without the addition of cytokines (Fig. 1). Although the presence of both GM-CSF and IL-4 was necessary for M $\phi$  to convert into MDDC, M $\phi$  treated with GM-CSF or IL-4 alone also underwent morphologic and phenotypic changes, becoming round and CD1a<sup>+</sup>CD14<sup>low</sup> (Fig. 1 and data not shown). A similar conversion pattern was found when CD14<sup>high</sup> cells cultured with M-CSF in Teflon dishes under NA conditions were purified by panning and recultured with GM-CSF/IL-4 (data not shown). Overall, most cells recovered from M-CSF cultures that had been subsequently switched to GM-CSF/IL-4 became CD1a<sup>+</sup> (Fig. 2A) with increased allostimulatory capacity in the MLR (Fig. 2B) and increased dextran uptake (2.8-fold higher MFI relative to M-CSF-treated cells), and they could differentiate into mature, strongly allostimulatory CD1a<sup>low</sup>CD83<sup>+</sup> DC upon LPS activation (Fig. 2, B and C).

Because culture of M-CSF-induced ADH M $\phi$  with GM-CSF/IL-4 consistently led to the appearance of two cell fractions, namely NA cells converting to DC and cells that remained ADH, we examined whether the latter fraction actually consisted of terminally differentiated M $\phi$ . To this end, we conducted bulk cultures of MO-enriched PBMC in Costar flasks ( $20 \times 10^6$  cells/20 ml) with M-CSF for 5 to 8 days, removed the NA cells, and added GM-CSF/IL-4 to the remaining ADH M $\phi$ . After 5 more days culture with GM-CSF/IL-4, two cell fractions were present, as seen previously. NA cells were collected, ADH cells were detached with EDTA-supplemented medium, and phenotypic evaluation revealed down-regulation of CD14 expression and the presence of 57% (range: 34–80%;  $n = 2$ ) and 54% (range: 22–71%;  $n = 3$ )



Table 1. Characteristics of M $\Phi$  and MDDC from MO-enriched PBMC cultured for 5 to 7 days with M-CSF or GM-CSF/IL-4, respectively<sup>a</sup>

	M $\Phi$	MDDC
Cell recovery <sup>b</sup>	$0.90 \pm 0.25 \times 10^6$ CD14 <sup>+</sup>	$0.95 \pm 0.40 \times 10^6$ CD1a <sup>+</sup>
Morphology <sup>c</sup>	Mixed: 1) ADH large, round and spindle-shaped cells; 2) minority of NA round, small cells (veiled in <7-day cultures)	Mostly NA or loosely ADH large cells with prominent dendrites and small clusters
Phenotype		
CD14 <sup>d</sup>	95 $\pm$ 5%	5 $\pm$ 5%
CD1a <sup>d</sup>	<1%	75 $\pm$ 27%
CD68 <sup>e</sup>	MFI index = 29	MFI index = 21
MPO	Negative at day 7	Low at day 5, negative at day 7
Function		
MLR <sup>f</sup> (cpm $\times 10^{-3}$ )	4.9 $\pm$ 3.7	33.3 $\pm$ 9.3
dextran uptake	+/-	++

<sup>a</sup> Data from  $\geq$  five independent experiments are given if not otherwise indicated.

<sup>b</sup> Mean  $\pm$  SD ( $n = 7$  and 6, respectively):  $2 \times 10^6$  cells were plated on day 0; only NA cells were counted in M-CSF cultures. No cell proliferation was detected (lack of 5-bromodeoxyuridine incorporation, data not shown).

<sup>c</sup> Evaluated with an inverted microscope.

<sup>d</sup> Mean  $\pm$  SD ( $n = 7$  and 6, respectively).

<sup>e</sup> Mean of two experiments: MFI of test sample/MFI of isotype-matched control.

<sup>f</sup> Responses of  $5 \times 10^4$  T lymphocytes to  $10 \times 10^3$  stimulating cells; mean  $\pm$  SD ( $n = 3$ ).

CD1a<sup>+</sup>CD14<sup>-</sup> cells with high allostimulatory capacity in each fraction, respectively (Fig. 2D).

#### Immature MDDC convert to M $\Phi$ in a M-CSF-independent manner

The in vitro instability of immature MDDC generated with GM-CSF/IL-4 has already been reported (11, 14, 17). We also found

that upon removal of both GM-CSF and IL-4 and/or reculture with M-CSF, day 5 CD1a<sup>+</sup>CD14<sup>-</sup> MDDC (the characteristics of which are summarized in Table 1) easily readhered and expressed membrane CD14 again (Fig. 3). In contrast to a previous report (11), this conversion was M-CSF independent (Fig. 3). Accordingly, we followed the kinetics of MDDC conversion to M $\Phi$  in cytokine-free cultures (Fig. 4). From day 2 to day 5 of culture without cytokines

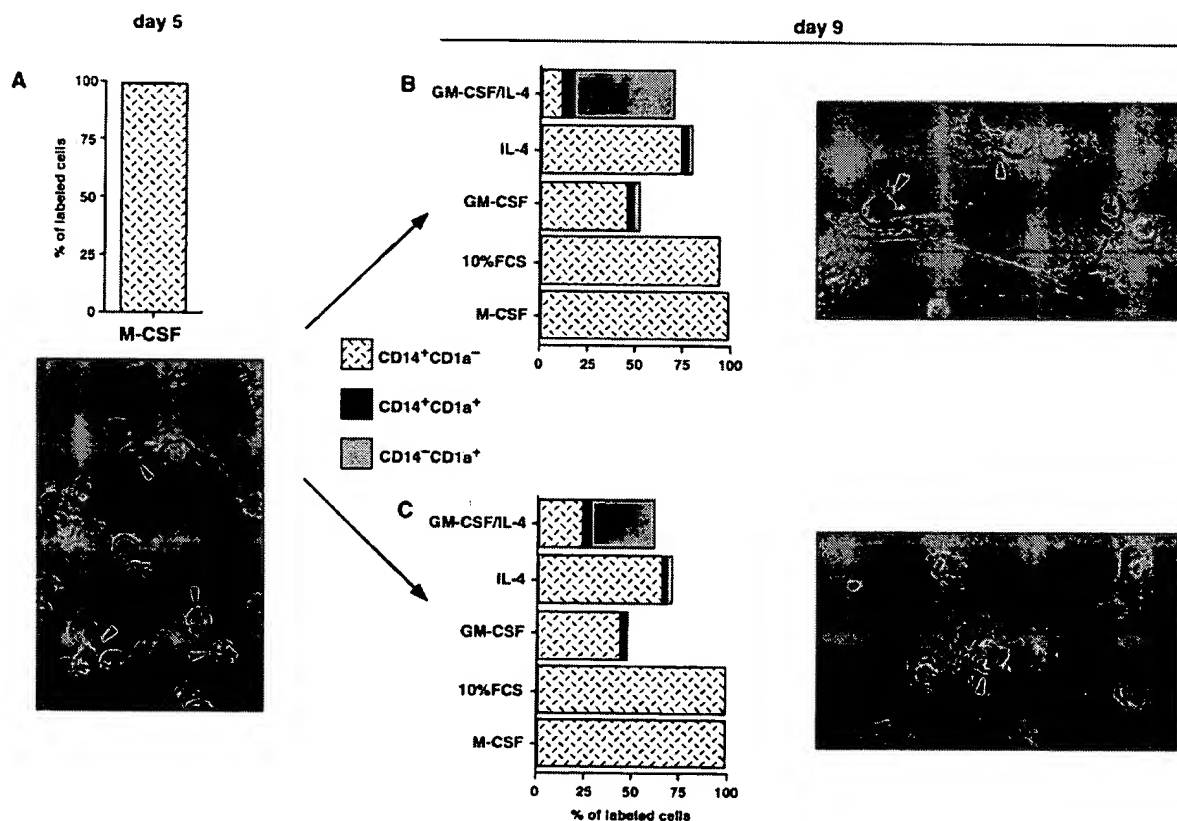
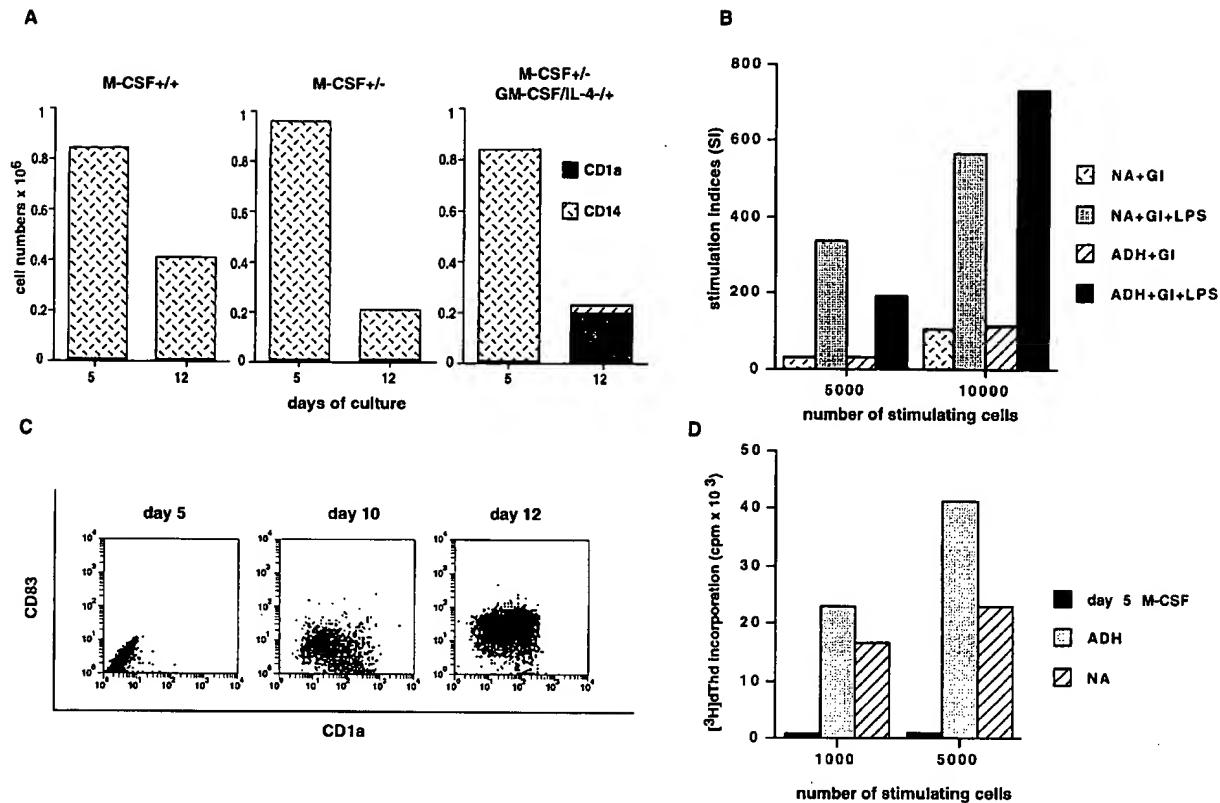


FIGURE 1. M-CSF-driven M $\Phi$  convert into MDDC in a GM-CSF/IL-4-dependent manner. NA and ADH cells of day 5 M-CSF cultures were separated and cultured for 4 more days under the indicated conditions. Cell percentages (plots) and cell morphology ( $\times 400$  magnification) were evaluated at the indicated time points. A, Cells of day 5 M-CSF cultures (arrows point to NA veiled cells). B, Culture of the day 5 NA fraction cells with GM-CSF/IL-4 (arrows point to DC). C, Culture of the day 5 ADH fraction cells with GM-CSF/IL-4 (the arrow points to DC). Cumulative data from five experiments are shown.



**FIGURE 2.** Characterization of the conversion of M $\phi$  to MDDC. *A*, CD1a<sup>+</sup> and CD14<sup>+</sup> cell counts at days 5 and 12 in culture with M-CSF (M-CSF+/+) or 7 days after the switch from M-CSF to either cytokine-free medium (M-CSF+/-) or GM-CSF/IL-4 (M-CSF+/-GM-CSF/IL-4/+). The first (+) and (-) indicates use or absence of the specified cytokine for the first 5 days, and the second sign refers to subsequent days; mean cell counts of five experiments starting from  $2 \times 10^6$  MO on day 0 are shown. *B*, MLR-stimulating capacity of immature and LPS-driven MDDC converted from M $\phi$  that were previously cultured for 7 days with M-CSF. Stimulating cells used were: NA+GI = day 7 NA cells cultured for 5 days with GM-CSF/IL-4; NA+GI+LPS = day 7 NA cells cultured for 5 days with GM-CSF/IL-4 and for 2 days with LPS; ADH+GI = day 7 ADH cells cultured for 5 days with GM-CSF/IL-4; ADH+GI+LPS: day 7 ADH cells cultured for 5 days with GM-CSF/IL-4 and for 2 days with LPS the responses of  $5 \times 10^4$  allogeneic T lymphocytes (SI) are shown. Background incorporation was 20 cpm. One experiment representative of three is shown. *C*, Phenotype of day 5 M-CSF-induced M $\phi$  cultured for an additional 5 days with GM-CSF/IL-4 and with LPS for the last 2 days: FITC-CD1a and phycoerythrin (PE)-CD83 labeling. *D*, ADH cells of day 5 bulk M-CSF cultures were cultured for 5 days with GM-CSF/IL-4; the resulting NA and ADH cells were evaluated for their capacity to stimulate  $5 \times 10^4$  allogeneic T lymphocytes (cpm) as compared with day 5 M $\phi$ . One experiment of two performed is shown.

(day 7 to day 10 of total culture), the MDDC progressively became ADH (Fig. 4A), and after  $\geq 5$  days,  $>50\%$  of cells had become round or spindle-shaped ADH M $\phi$  (Fig. 3B). Only 2 days after cytokine removal, cells of the majority of donors reexpressed CD14 but remained CD1a<sup>+</sup>, with  $76 \pm 14\%$  being CD1a<sup>+</sup>CD14<sup>-</sup> and  $21 \pm 14\%$  being CD1a<sup>+</sup>CD14<sup>+</sup> (Fig. 4B). However, after  $\geq 5$  days, even the cells that remained NA in cytokine-free culture displayed a CD1a<sup>low/-</sup>CD14<sup>high</sup>MPO<sup>-</sup> M $\phi$  phenotype (Fig. 4B and data not shown) with a decreased ability to stimulate allogeneic T cells (Fig. 4, C and D) and take up dextran (40% lower MFI relative to cells treated continuously with GM-CSF/IL-4).

#### The conversion potential to M $\phi$ is a true property of CD1a<sup>+</sup> MDDC

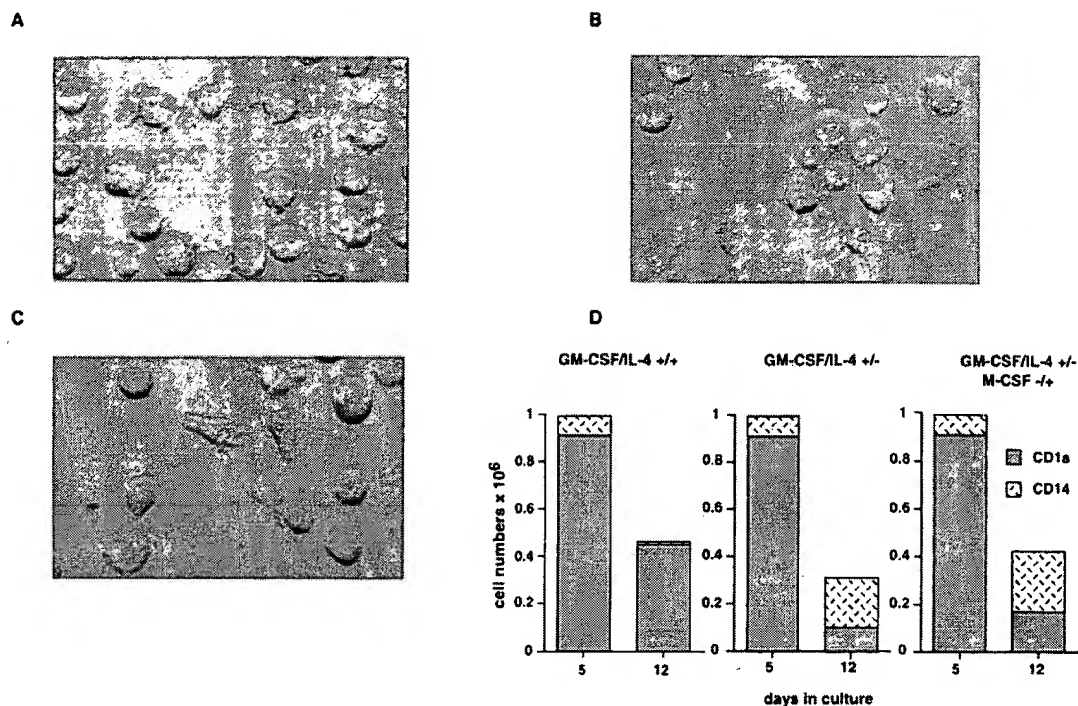
Next, we examined whether the changes in MDDC morphology, phenotype, and function described above were due to true conversion or whether they were the consequence of the heterogeneity of the day 5 cultured cells instead, resulting in the selection of a particular cell type upon cytokine withdrawal. To answer this question, CD1a<sup>high</sup> MDDC purified to  $>95\%$  by panning (data not shown) or FACS sorting (Fig. 5) were cultured with either GM-CSF/IL-4 or M-CSF. Again, the majority of cells converted to M $\phi$

phenotype and morphology (Fig. 5). Moreover, MDDC that had converted to ADH M $\phi$  could again acquire CD1a expression and DC morphology upon reculture with GM-CSF/IL-4 (Fig. 5).

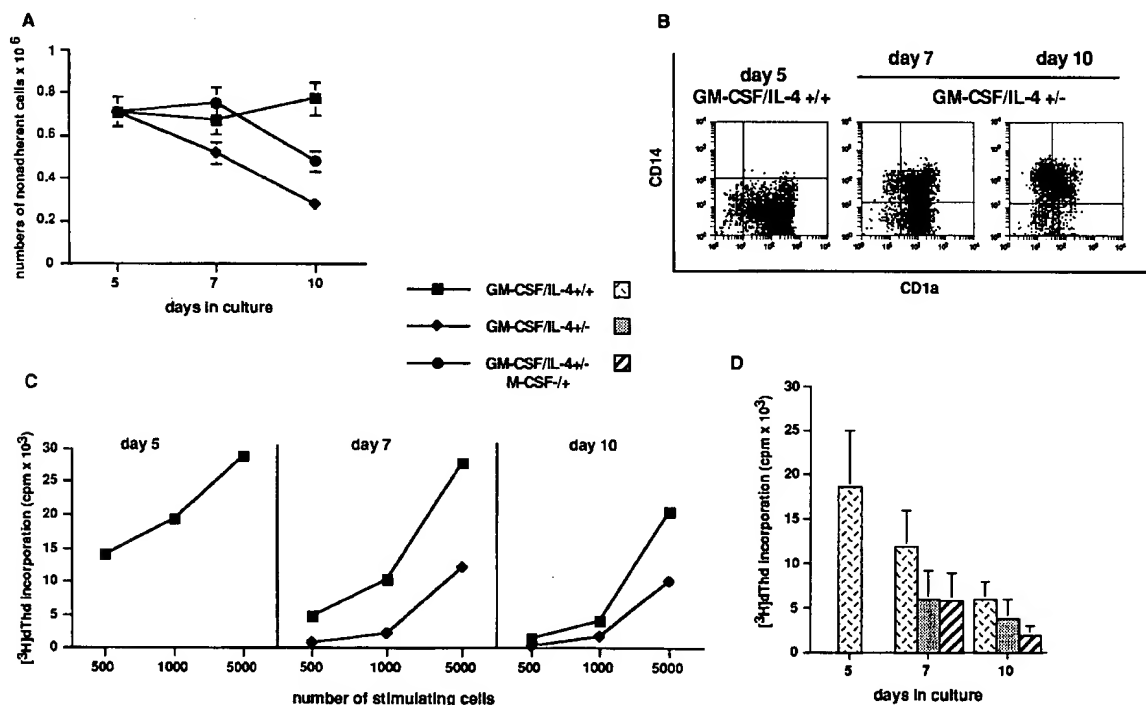
#### Mature CD1a<sup>+</sup>CD83<sup>high</sup> MDDC cannot convert to M $\phi$

MDDC maturation, as assessed by morphology and phenotype, was induced by culturing day 5 MDDC for  $>2$  days with LPS, CM, or TNF- $\alpha$ /IL-1 $\beta$ ; all of these conditions led to the induction of CD83 expression, increased the expression of costimulatory molecules, and increased the allostimulatory capacity of cells relative to control cultures (Table II and Fig. 6, A and B). Mature MDDC were then cultured in cytokine-free medium or with M-CSF. LPS-treated MDDC remained CD1a<sup>+</sup>CD14<sup>-</sup>CD83<sup>+</sup> (data not shown) or died in the new environment (70–80% PI<sup>+</sup> cells). Similarly, CM-treated MDDC remained mostly NA and CD1a<sup>low/-</sup>CD14<sup>-</sup>CD83<sup>low</sup> (Fig. 6, B and C), confirming the results of others (17). We took advantage of the fact that TNF- $\alpha$ /IL-1 $\beta$ -driven MDDC displayed heterogeneous CD83 expression to sort them into CD83<sup>high</sup> and CD83<sup>low/-</sup> cells (Fig. 7A). Sorted CD1a<sup>+</sup>CD83<sup>high</sup> cells displayed much higher allostimulatory capacity than CD1a<sup>+</sup>CD83<sup>low/-</sup> cells from the same cultures (Fig. 7B). Upon cytokine removal (data not shown) or in culture with

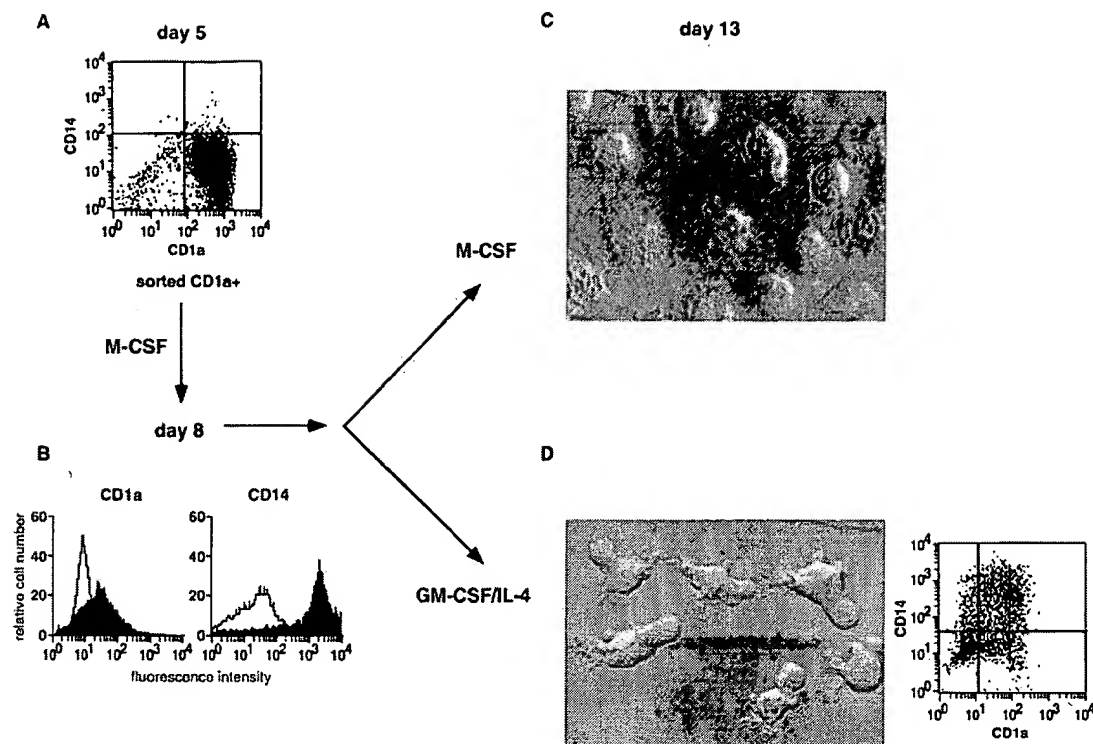




**FIGURE 3.** M-CSF-independent conversion of immature MDCC into M $\phi$ . *A*, Morphology of cells cultured for 12 days with GM-CSF/IL-4; the conversion of MDCC to ADH M $\phi$  upon cytokine withdrawal (*B*) or replacement with M-CSF from culture day 5 to 12 (*C*) ( $\times 400$  magnification) is shown. *D*, CD1a<sup>+</sup> and CD14<sup>+</sup> cell counts at days 5 and 12 in culture with GM-CSF/IL-4 (GM-CSF/IL-4+/+) or 7 days after switching from GM-CSF/IL-4 to either cytokine-free medium (GM-CSF/IL-4+/-) or M-CSF (GM-CSF/IL-4+/-M-CSF-/+). Cumulative data from six experiments (mean cell counts starting from  $2 \times 10^6$  day 0 plated MO) are shown; see legend to Figure 2 for definition of (+) and (-).



**FIGURE 4.** Kinetics of the conversion of MDCC into M $\phi$ . *A*, Recovered NA cell numbers after culture under the indicated conditions (see legend to Fig. 2) starting from  $2 \times 10^6$  day 0 plated MO; mean values  $\pm$  SD ( $n = 5$ ) are given. *B*, Kinetics of CD1a and CD14 expression by cells cultured under the indicated conditions, as seen with two-color cytograms of cells labeled with FITC-CD1a and PE-CD14 mAbs; the horizontal and vertical lines indicate the level of autofluorescence in the FL-2 and FL-1 channels. Data from one representative experiment of five is shown. *C*, MLR-stimulating capacity for  $5 \times 10^4$  allogeneic T lymphocytes (cpm) of the NA cells from the same culture seen in *B*. *D*, Kinetics of the MLR-stimulating capacity for  $5 \times 10^4$  T lymphocytes (cpm) of  $1 \times 10^3$  NA cells cultured under the indicated conditions; mean  $\pm$  SD ( $n = 3$ ) is given.



**FIGURE 5.** The conversion potential of CD1a<sup>+</sup> MDDC to Mφ and back. Mφ obtained from the conversion of day 5 MDDC cultured with M-CSF convert back into CD1a<sup>+</sup> DC upon reculture with GM-CSF/IL-4. Two-color cytograms of day 5 (*A*) and day 13 (*D*) cells cultured under the indicated conditions and labeled with FITC-CD1a and PE-CD14 mAbs are shown; the horizontal and vertical lines are as defined in the legend to Figure 4. *B*, Day 8 CD1a and CD14 labeling of cells cultured with GM-CSF/IL-4 for 5 days and then with M-CSF for 3 days. Open histograms indicate control labeling with an irrelevant mAb, while solid histograms indicate staining by the relevant mAb. *C*, Day 13 morphology of day 8 NA cells cultured with M-CSF. *D*, Day 13 morphology of day 8 ADH cells cultured with GM-CSF/IL-4 (×400 magnification). Combined results from two of three experiments are shown.

M-CSF, most CD83<sup>low/-</sup> cells converted to Mφ (with <10% PI<sup>+</sup> cells) (Fig. 7*D*); CD83<sup>high</sup> cells either remained NA CD1a<sup>+</sup>CD14<sup>-</sup> DC or died (>45% PI<sup>+</sup> cells) but did not convert to Mφ (Fig. 7*C*).

## Discussion

The blood is a reservoir from which MO can be recruited into tissues and differentiate into Mφ and/or APC (20). Our data

support the idea of MO being relatively immature precursors with a double differentiation potential that depends upon the cytokine environment. Indeed, MO-enriched PBMC (>80% CD14<sup>+</sup>CD68<sup>+</sup>MPO<sup>low</sup>) cultured with either M-CSF or GM-CSF/IL-4 differentiated into Mφ (CD14<sup>high</sup>CD1a<sup>-</sup>CD68<sup>+</sup>MPO<sup>-</sup>) or MDDC (CD14<sup>low/-</sup>CD1a<sup>high</sup>CD68<sup>+</sup>MPO<sup>-</sup>), respectively. The majority of NA and loosely ADH M-CSF-driven Mφ could convert into MDDC if cultured anew with both GM-CSF and

**Table II.** Characteristics of MDDC cultured for 5 days with GM-CSF/IL-4 and cultured further for 2 days with maturation-inducing factors (7 days total culture)<sup>a</sup>

	TNF-α/IL-1β	LPS	CM
Cell recovery <sup>b</sup>	0.40 ± 0.15 × 10 <sup>6</sup>	0.75 ± 0.30 × 10 <sup>6</sup>	1.00 ± 0.20 × 10 <sup>6</sup>
Morphology <sup>c</sup>	Heterogeneous clustered and nonclustered cells, partly ADH	Strongly clustered cells with prominent dendrites	"Hairy" cells with abundant short dendrites
Phenotype: <sup>d</sup>			
CD1a <sup>+</sup>	67-94% <sup>low/high</sup>	>85% <sup>low/high</sup>	79-98% <sup>low</sup>
CD1a <sup>+</sup> CD83 <sup>+</sup>	Heterogeneous: 9-33% <sup>high</sup> and 38-67% <sup>low</sup>	Mostly homogenous: >90% CD83 <sup>+</sup>	Low CD83 expression on all cells
CD80 <sup>+</sup> CD86 <sup>+</sup>	20 ± 7%	84 ± 19%	75 ± 10%
HLA-DR	>90% <sup>low/high</sup>	>90% <sup>high</sup>	>90% <sup>low/high</sup>
MLR index <sup>e</sup>	1.2	1.9	1.2

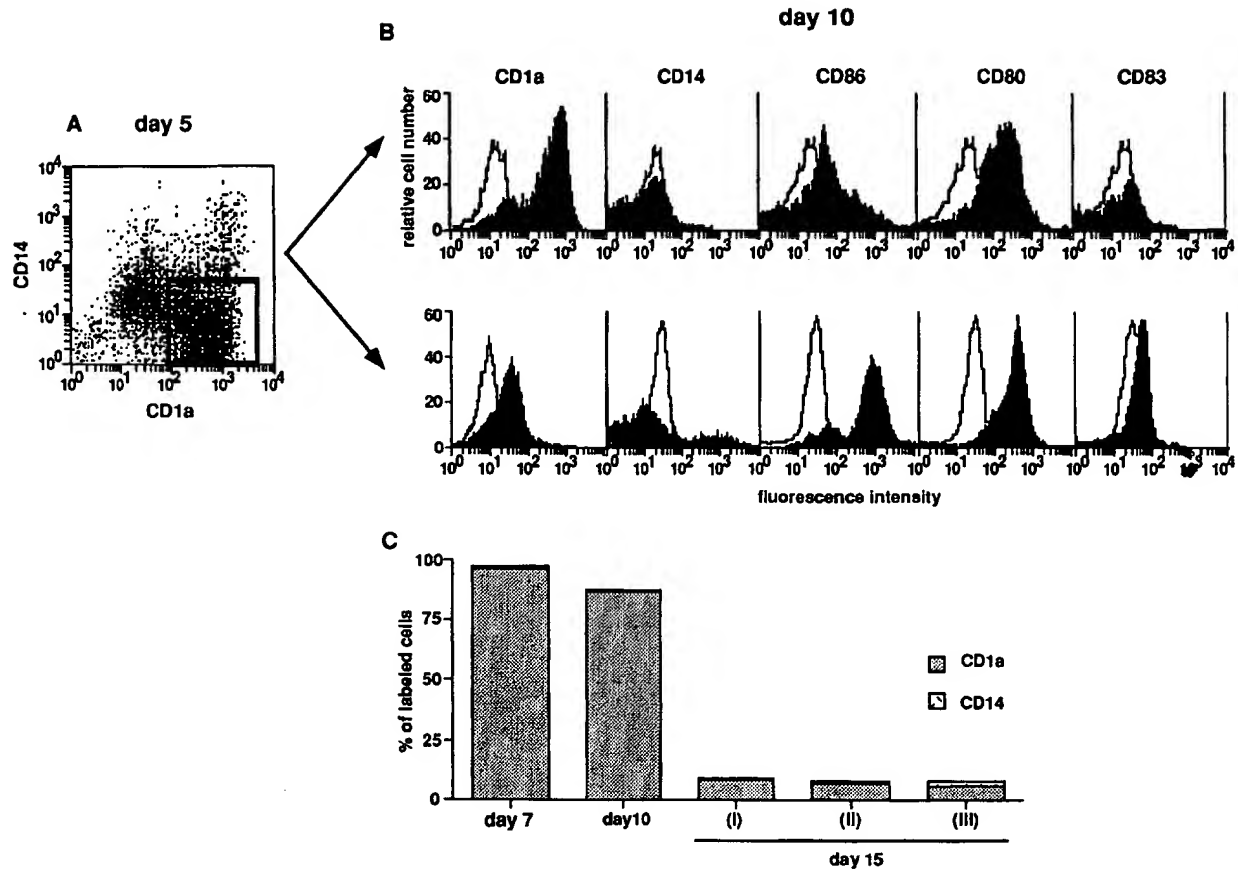
<sup>a</sup> Data from ≥ three independent experiments are given if not otherwise indicated.

<sup>b</sup> Mean ± SD (*n* = 5); 2 × 10<sup>6</sup> cells were plated on day 0.

<sup>c</sup> Evaluated with an inverted microscope; some adherent cells resulting from GM-CSF/IL-4 removal could be seen in all cultures;

<sup>d</sup> Percentage of positive cells is shown (*n* = 5).

<sup>e</sup> Allo-MLR response induced by day 7 mature MDDC relative to day 7 immature MDDC cultured with GM-CSF/IL-4 only in the same experiment (ratios of cpm); the response of 5 × 10<sup>4</sup> T lymphocytes to 5 × 10<sup>3</sup> stimulating cells is shown and data are representative of three experiments.



**FIGURE 6.** MDDC induced to maturation by CM do not convert into M $\phi$  if cultured under switched cytokine condition or in cytokine-free medium. *A*, A two-color cytogram of day 5 MDDC labeled with FITC-CD1a and PE-CD14 mAbs showing the CD1a<sup>+</sup> cell sort gate. *B*, Histograms illustrate the phenotype of CD1a-sorted MDDC cultured for 5 more days with GM-CSF/IL-4 (*upper panels*) or CM (*lower panels*). Open histograms indicate control labeling with an irrelevant mAb, while solid histograms indicate staining with the relevant mAb against the indicated marker. *C*, Cells cultured in GM-CSF/IL-4 for 7 days (day 7) and then with CM for 3 days (day 10) were collected and recultured for 5 more days (day 15) with CM (I), or in cytokine-free medium (II), or with M-CSF (III); percentages of CD1a<sup>+</sup> and CD14<sup>+</sup> cells in the populations recovered at the indicated time point are shown. Combined results from two of four experiments are shown.

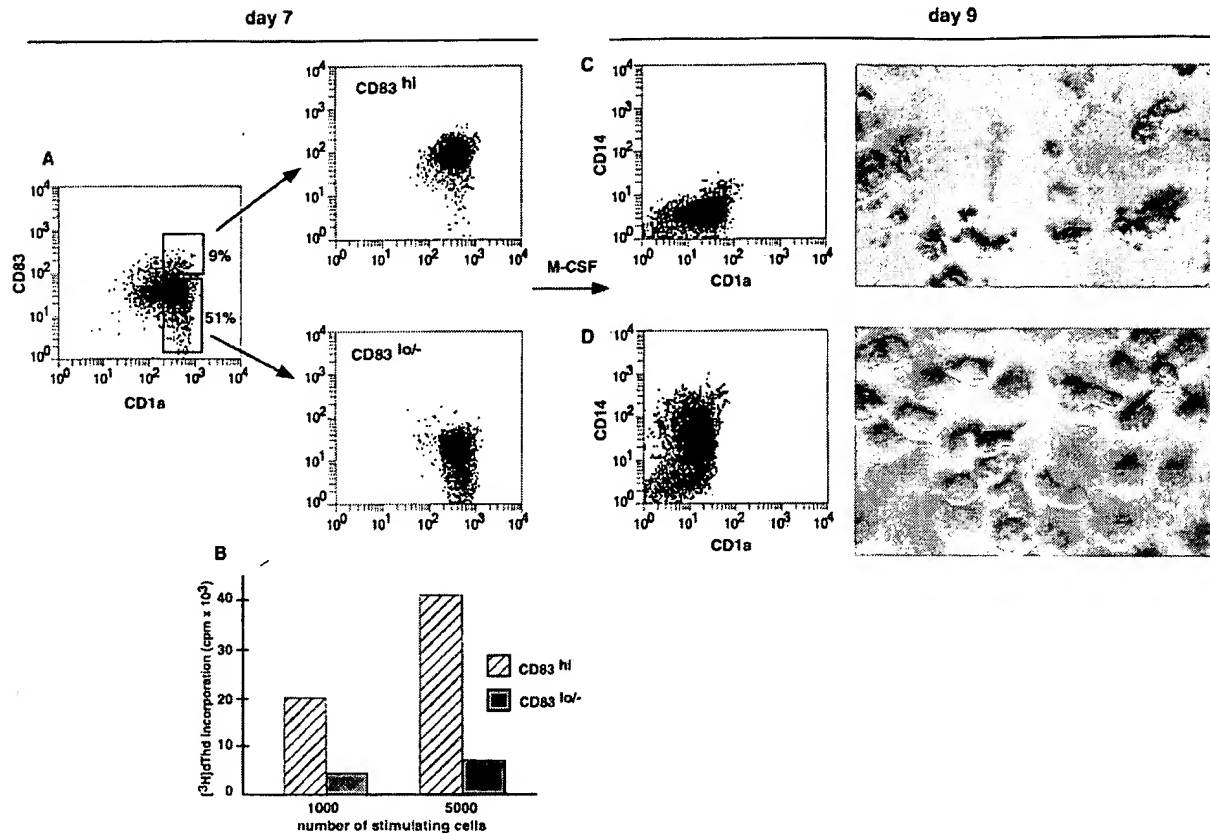
IL-4 as determined by morphologic, phenotypal, and functional criteria. The conversion potential of strongly ADH M $\phi$  varied depending upon the culture condition; while the majority of ADH cells generated in Costar plates appeared irreversibly committed, >50% of ADH M $\phi$  generated in bulk cultures readily converted to MDDC. Moreover, even cells that remained ADH in the presence of GM-CSF/IL-4 did acquire CD1a expression and were capable of stimulating allogeneic T cells.

The necessity of both GM-CSF and IL-4 for the conversion of M $\phi$  to MDDC to occur is in line with our earlier results showing that both cytokines are needed for MO to differentiate into DC (15). GM-CSF is known to down-regulate CD14 expression at the transcriptional level in MO (21), and this effect is potentiated by IL-4 (22). The previously reported formation of multinucleated giant cells when IL-4 was added to M-CSF-driven M $\phi$  (11, 23) was not a major phenomenon in our cultures, although we could sporadically observe cell fusions with multinucleated cells that never involved more than three nuclei (data not shown).

Alternatively, immature MDDC converted to CD14<sup>low/-</sup>CD1a<sup>low/-</sup> MPO<sup>-</sup> M $\phi$  upon cytokine removal, regardless of the presence of M-CSF. However, in some donors, adding M-CSF could hasten CD14 reexpression and increase the proportion of ADH spindle-shaped cells. In another study, the conversion of MDDC to M $\phi$  was ascribed to M-CSF, since the cells died upon GM-CSF/IL-4 with-

drawal without cytokine replacement (11). We observed neither selective death of MDDC cultured in cytokine-free medium nor M-CSF rescue of cells, and the total numbers of recovered CD1a and/or CD14 cells were similar under both culture conditions. The plausible explanation for this discrepancy could be the different MO-enrichment and culture methods used in each study. It has been shown that upon adhesion-dependent activation, MO can be induced to produce various cytokines (reviewed in Ref. 20) and, consequently, MDDC generated from nondepleted ADH PBMC (11) may have different growth requirements than MDDC generated from lymphocyte-depleted MO-enriched PBMC.

Interestingly, MDDC driven to maturation by TNF- $\alpha$ /IL-1 $\beta$  were heterogeneous with regard to CD83 expression, and all were not terminally differentiated, as most of CD83<sup>low</sup> cells easily converted to M $\phi$  in cultures with M-CSF. Whether irreversible MDDC maturation is a random process or only involves MDDC subsets primed for terminal differentiation and maturation remains to be determined. In our study, day 5 cells of GM-CSF/IL-4 cultures were heterogeneous with regard to CD86 expression; and there consistently were  $\leq 15\%$  CD1a<sup>+</sup>CD86<sup>+</sup> MDDC, which could represent a cell subset primed for terminal maturation. Nevertheless, our results support the current concept that DC require a second signal (optimally CD40 ligation) for terminal maturation (our manuscript in preparation and (5, 8, 24, 25)). In contrast to



**FIGURE 7.** The heterogeneity of MDDC pushed to maturation by  $\text{TNF-}\alpha/\text{IL-1}\beta$ . This experiment is representative of three performed. Day 7  $\text{CD83}^{\text{high}}$  and  $\text{CD83}^{\text{low/-}}$  cells from the culture with GM-CSF/IL-4 for 5 days followed by 2 days with  $\text{TNF-}\alpha/\text{IL-1}\beta$  were sorted to >95% purity and cultured further with M-CSF; NA  $\text{CD83}^{\text{high}}$  cells remained  $\text{CD1a}^+\text{CD14}^-$ , while  $\text{CD83}^{\text{low/-}}$  cells readily converted to M $\phi$ . A two-color cytogram of day 5 MDDC labeled with FITC-CD1a and PE-CD83 mAbs is shown. *A* indicates the sort windows and the phenotype of the resulting sorted populations. *B* indicates the MLR-stimulating capacity for  $5 \times 10^3$  allogeneic T lymphocytes (cpm) of day 7-sorted  $\text{CD83}^{\text{high}}$  and  $\text{CD83}^{\text{low/-}}$  cells. Two-color cytograms and morphology ( $\times 400$  magnification) of day 9 cells from the culture of sorted  $\text{CD83}^{\text{high}}$  (*C*) and  $\text{CD83}^{\text{low}}$  (*D*) cells labeled with FITC-CD1a and PE-CD14 mAbs are shown.

$\text{TNF-}\alpha/\text{IL-1}\beta$ -induced MDDC, only part of which were terminally differentiated, MDDC driven to maturation by LPS (a physiologic "danger" signal (26)) or by in vitro-produced MO-CM could not revert to M $\phi$  in the new cytokine environment.

Since prolonged culture resulted in substantial cell loss, it may be argued that the potential of M $\phi$  to convert to MDDC and vice versa is confined to only a fraction of both cell types that are progressively selected in the course of experiments. However, it seems more likely that cell loss (noted under all conditions) is random and due to the experimental manipulation of highly activated cells. This would be particularly true for the ADH cell fractions, the detachment of which results in high mortality that may lead to a possible underestimation of cell counts.

Our results show that M $\phi$  and MDDC can readily interconvert into one another until the late stages of their respective differentiation/maturation process. Thus, it is conceivable that in a situation in which exogenous or endogenous tissue damage is beyond the capability of first-line innate immunity for in situ repair, resident M $\phi$  will convert to DC upon appropriate signals and travel to regional lymph nodes to present the Ag and induce acquired immunity responses. This could be particularly true in a situation in which no immunologic memory is available for an Ag, thus making the recruitment of naive T and/or B cells necessary. Indeed, it

has recently been postulated that DC constitute a link between innate and acquired (adaptive) immunity (1, 2).

The concept of alternative MO developmental pathways leading either to "accessory" cells or M $\phi$  was already proposed in the mid-eighties (27). It was then observed, however, that M $\phi$  development is preceded by a transient differentiation stage of veiled cells with increased accessory function (27). It was not possible under any conditions for M $\phi$  to proceed through their development pathway without this stage, but they could remain at the veiled cell stage if appropriate differentiation signals were provided (27). Indeed, we have seen veiled cells that preceded adherence and the development of M $\phi$  morphology at early time points (up to day 7) in all M-CSF cultures. Veiled cells could also be seen early on (during the first 2–3 days) in GM-CSF/IL-4 cultures, and the allostimulatory capacity of such early cells was rather low, as previously reported (15). Taken together with the data presented here, these findings suggest that MO are immature precursors that go through a stage of veiled cells (immediate DC precursors), the ultimate fate of which is to become DC (Fig. 8). Accordingly, the local cytokine environment and the presence of maturation and other stimulatory signals may well be the final decision-making factors determining whether the cells will acquire DC or M $\phi$  characteristics and function.

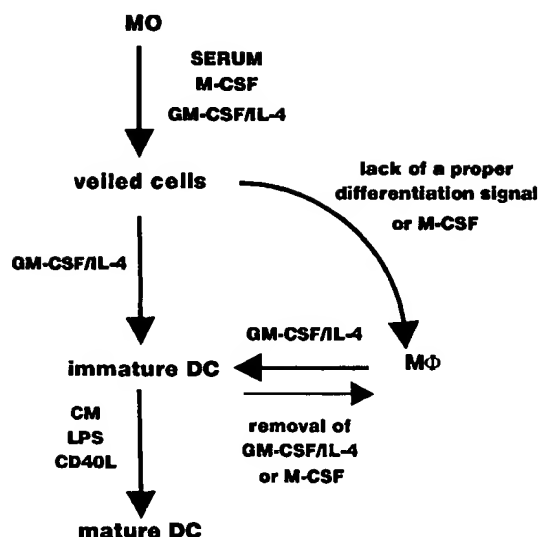


FIGURE 8. A model of the MO differentiation pathway.

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May be site of binding of Hepatitis C virus

**Positive staining (normal):** lymphocytes, endothelial cells, epithelial cells

**Negative staining:** erythrocytes, platelets, neutrophils

## CD82

### top

Aka prostate cancer antimetastasis gene KAI1, 'kang ai' (Chinese for anticancer).

Metastasis suppressor gene; downregulated in tumor progression of cancers

Expression correlates with p53 expression

Associates with CD4 or CD8 and delivers costimulatory signals for T cell receptor pathway

Activation antigen for T cells

**Positive staining (normal):** activated/differentiated hematopoietic cells

**Negative staining:** erythrocytes

**Reference:** [600623](#)

## CD83

### top

**Summary:** Marker for dendritic cells

May assist in antigen presentation or cellular interactions that follow lymphocyte activation.

**Positive staining (normal):** dendritic cells, Langerhans cells, lymphocytes

**Positive staining (disease):** Churg-Strauss syndrome myocarditis in inflammatory infiltrates, [Archives 2003;127:98](#)

**Micro images:** [Churg-Strauss syndrome myocarditis](#)

**Reference:** [GeneCards](#)

## CD84

### top

**Summary:** Homophilic adhesion molecule that enhances IFN-gamma secretion

**Positive staining (normal):** B cells, thymocytes, T cell subset, monocytes/macrophages, platelets

**Reference:** [GeneCards](#)

## SCIENTIFIC REVIEW

### Dendritic cells (I) : biological functions

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<u>Introduction</u>	<u>DC death (apoptosis)</u>
<u>Dendritic cell development</u>	<u>Dendritic cell distribution</u>
<u>Functional differences between the different DC subsets.</u>	<u>References</u>
<u>Migration and functional properties of dendritic cells</u>	

Dendritic cells (DCs) are potent antigen presenting cells (APCs) that possess the ability to stimulate naïve T cells. They comprise a system of leukocytes widely distributed in all tissues, especially in those that provide an environmental interface. DCs possess a heterogeneous haemopoietic lineage, in that subsets from different tissues have been shown to possess a differential morphology, phenotype and function. The ability to stimulate naïve T cell proliferation appears to be shared between these various DC subsets. It has been suggested that the so-called myeloid and lymphoid-derived subsets of DCs perform specific stimulatory or tolerogenic function, respectively. DCs are derived from bone marrow progenitors and circulate in the blood as immature precursors prior to migration into peripheral tissues. Within different tissues, DCs differentiate and become active in the taking up and processing of antigens (Ags), and their subsequent presentation on the cell surface linked to major histocompatibility (MHC) molecules. Upon appropriate stimulation, DCs undergo further maturation and migrate to secondary lymphoid tissues where they present Ag to T cells and induce an immune response. DCs are receiving increasing scientific and clinical interest due to their key role in anti-cancer host responses and potential use as biological adjuvants in tumour vaccines, as well as their involvement in the immunobiology of tolerance and autoimmunity.

Keywords: antigens, dendritic cells, development, distribution, functions, maturation, T cells

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#### INTRODUCTION

Dendritic cells (DCs), originally identified by Steinman and his colleagues (1972) represent the pacemakers of the immune response.<sup>1</sup> They are crucial to the presentation of peptides and proteins to T and B lymphocytes and are widely recognized as the key antigen presenting cells (APCs). They are critical for the induction of T cell responses resulting in cell-mediated immunity (CMI). The T cell receptors (TCRs) on T lymphocytes recognize fragments of antigens (Ags) bound to molecules of the major histocompatibility complex (MHC) on the surfaces of APCs. The peptide binding proteins are of two types, MHC class I and II, which interact with and stimulate cytotoxic T lymphocytes (CTLs) and T helper cells (Ths), respectively. On entry into APCs, Ags are processed, spliced into peptides in the cytosol and then reexpressed on the cell surface linked to MHC proteins. When bound to MHC class I molecules, CTLs are generated and activated and cells in tissues expressing the Ags (e.g. virus infected cells, cancer cells) are recognised and destroyed. Antigens reexpressed on the cell surface linked to MHC class II

molecules interact with Th cells which when activated have profound immune-regulatory effects.<sup>2</sup> Thus, DCs play a key role in host defenses and a crucial role in putative anti-cancer immune responses.

## DENDRITIC CELL DEVELOPMENT

### Introduction

An important advance in DC biology, within the past few years, has been the ability to propagate in vitro large numbers of DCs, using defined growth factors. One of the most important findings is that DCs are not a single cell type, but a heterogeneous collection of cells that have arisen from distinct, bone marrow-derived, hematopoietic lineages.<sup>3-7</sup> To date, at least three different pathways have been described. The emerging concepts are that each pathway develops from unique progenitors, that particular cytokine combinations drive developmental events within each pathway and that cells developing within a particular pathway exhibit distinct specialized functions.<sup>3-7</sup>

The ability to propagate DC subtypes, at various stages of development in vitro, from early progenitors has been critical in assessing the developmental and functional characteristics of DCs. Together with in situ histochemical analyses and genetically modified animal models, in vitro studies have shown that the earliest DC progenitors/precursors are released from the bone marrow and circulate through the blood and lymphoid organs ready to receive differentiation signals.<sup>4-6</sup>

Several studies have been carried out suggesting that there are different pathways for the formation of mature DCs from CD34+ or other primitive progenitors. Each pathway differs in terms of progenitors and intermediate stages, cytokine requirements, surface marker expression and, probably most importantly, biological function.

### Lymphoid-related DC pathway

The close lineage relationship of DCs and monocytes has been challenged in recent years by the findings of several groups who have described the development of DCs and lymphoid cells from the same precursors. The term lymphoid DC is meant to describe a distinct DC subtype that is closely linked to the lymphocyte lineage.

Initially described in the mouse, the term 'lymphoid' refers to several features that suggest a precursor in common with T cells. This pathway appears to lack a number of characteristics found in myeloid cells, in particular, lack of defined surface phenotypes-CD11b, CD13, CD14, and CD33. <sup>7</sup> In blood, the lymphoid precursors may be the CD4+ CD11c+ 'plasma-like cell'. There is now some evidence that an equivalent lymphoid lineage DC exists in humans.<sup>7-12</sup> Lymphoid DCs may also arise from progenitors that also have the potential to mature into T and natural killer (NK) cells.<sup>7-12</sup> Such progenitors are distributed in the thymus and in the T cell areas of secondary lymphoid tissues.<sup>5,7-12</sup> Lymphoid DCs may develop from thymic progenitors when stimulated with Interleukin 3 (IL-3), but not granulocyte macrophage colony-stimulating factor (GM-CSF), and from lymphoid precursors in human tonsil treated with CD40 ligand (CD40L).<sup>7,10</sup> More recently, IL-2 and IL-15 have been shown to drive NK cell-associated (IL-2R+) DCs from CD34+ progenitors.<sup>11,12</sup>

A variety of functions have been attributed to lymphoid DCs. They promote negative selection in the thymus (possibly by inducing fas-mediated apoptosis) and are costimulatory for CD4+ and CD8+T cells.<sup>7, 13-15</sup> More recently, lymphoid-like DCs derived from human progenitors have also been shown to preferentially activate the Th2 response.<sup>14</sup> Because of their capacity to induce apoptosis and their role in eliminating potentially self-reactive T cells, it has been suggested that lymphoid DCs primarily mediate regulatory rather than stimulatory immune effector functions.<sup>7, 14, 16</sup>

In the human bone marrow, Galy et al (1995) identified a subset of progenitor cells defined by the phenotype CD34+ CD38+ Thy-1- CD10+. The latter, when cultured under appropriate conditions, were capable of giving rise



to T, B, NK and DCs but not to myeloid cell types.<sup>8</sup> Progenitors with a similar phenotype but lacking CD10 expression could give rise to myeloid cells and more prolonged thymopoiesis suggesting that acquisition of CD10 corresponded to a maturation step and commitment to T, B, NK and DC lineages. Similarly, a CD34<sup>+</sup> Thy-1<sup>-</sup> but CD38<sup>dim</sup> foetal thymic precursor gave rise to T, NK and DCs but not other (myeloid) lineages, whilst the more mature CD34<sup>+</sup> CD38<sup>+</sup> thymic precursor was shown to have less DC potential.<sup>17</sup> These results suggest that thymic DCs and thymocytes are derived from a common precursor that migrates to the thymus prior to lineage commitment and terminal differentiation. Lymphoid DCs include those in the thymic medulla and many of the DCs in the T cell areas of all peripheral lymphoid organs. Dendritic cells in the latter T cell areas, however, are heterogeneous and include other types of DCs, for example, sentinel and migratory DCs that have brought Ags from peripheral tissues. Lymphoid DCs in T cell areas have the capacity to regulate self-reactive T cells, for example, by the production of IL-10 or other cytokines, or to delete them, for example, by induction of apoptosis by a member of the tumour necrosis factor (TNF) family like fasL<sup>18</sup> or CD30L.<sup>19</sup>

### **Myeloid DC pathway: CD34<sup>+</sup> haemopoietic progenitors**

The myeloid pathway is distinguished by a development stage in which there is expression of certain features associated with phagocytes. Studies with multipotent CD34<sup>+</sup> progenitors and peripheral blood mononuclear cells (PBMCs) have described different DC pathways, both associated with the myeloid lineage.

The skin contains a prominent supply of tissue DCs, termed Langerhan cells (LCs), which have typical DC morphology and contain characteristic Birbeck granules (BGs), seen on electron microscopy or by staining, using a specific monoclonal antibody (Mab).<sup>20</sup> Evidence from murine bone marrow transplantation studies suggests that LCs are derived from the donor and that they are presumably of myeloid origin.<sup>21</sup> In the rat, Bowers and Berkowitz (1986) demonstrated DCs in myeloid colonies in semi-solid cultures of Ia<sup>-</sup> bone marrow precursors and this was noted also in clonal assays of human bone marrow.<sup>22,23</sup> More recent studies, using methylcellulose culture assays of human bone marrow or PBMCs, identified colonies both of pure DCs and also of mixed dendritic/macrophage cell types. Colonies were observed after 14 days of culture when stimulated by leukocyte conditioned medium.<sup>24</sup> The resultant cells resembled LCs in their gross morphology and ultrastructure though they lacked the BGs typical of skin LCs. They were CD34<sup>+</sup>, had high levels of HLA-DR but, unlike the macrophages from the same cultures, were also HLA-DQ<sup>+</sup> and lacked both a strong non-specific esterase expression and the monocyte-associated cytoplasmic antigen CD68. Most characteristically, they strongly expressed CD1a<sup>+</sup> and their DC phenotype was confirmed by high allostimulatory activity in mixed leukocyte reactions (MLRs)-greater than either macrophages or even fresh blood DCs.

### **Myeloid DC pathway: PBMCs and CD14<sup>+</sup> cells**

There is considerable evidence from culture studies for a close developmental relationship between DCs and cells of the monocyte/macrophage lineage.<sup>6,25</sup> Adherent PBMCs are enriched for monocytes, and this fraction may develop a LC phenotype and function if cultured in the presence of foetal calf serum (FCS).<sup>26,27</sup> Further, amongst the PBMCs, only purified monocytes are capable of expressing the LC marker CD1a if cultured in GM-CSF.<sup>28</sup> The cytokines required for the in vitro production of DCs from the adherent fraction of PBMCs were first documented by Romani et al (1994) and by Sallusto and Lanzavecchia (1994).<sup>29,30</sup> They demonstrated that cultures of PBMCs in GM-CSF and IL-4 produced cells that were CD1a<sup>+</sup> CD14<sup>-</sup> and capable of Ag uptake and processing, the typical profile of immature DCs. Yields of up to 8x10<sup>6</sup> DCs were obtained from 40 ml of blood.<sup>29,30</sup> The possibility that the DCs were derived from contaminating progenitor cells had been excluded by using highly purified CD14<sup>Bright</sup> monocytes<sup>31</sup> and the absence of cellular proliferation in culture.<sup>20, 32, 33</sup> Although the resulting cells resembled immature DCs they were atypical because of the presence of lysozyme, myeloperoxidase, non-specific esterase and their lack of CD83.<sup>20, 21, 31</sup> In some studies, further differentiation into fully mature DCs could be induced by exposing these cells to a or CD40.<sup>21</sup> This final maturation was characterised by downregulation of the ability to

take up and process Ag whilst CD54, HLA-DR, CD83 and CD80 expression increased in parallel with the Ag presenting function.<sup>21</sup> Rozenwaig et al. (1996) demonstrated a well ordered phenotypic evolution of DC precursors via CD13Low progenitors to CD13High CD1a- and then CD13High CD1a+ intermediates that also expressed variable levels of CD14.<sup>23</sup> Using a similar approach, two pathways of DC maturation from cord blood CD34+ progenitors were identified. After 5 days in culture with GM-CSF, SCF and TNF- $\alpha$ , cells were sorted into either CD14+CD1a- or CD14-CD1a+ populations (see Table 1).<sup>3</sup>

**Table 1:** Comparison of the different developmental pathways of DCs of myeloid origin

Derived from CD14+CD1a -	Derived from CD14-CD1a+
Related to interstitial and/or circulating blood DCs	Related to epidermal DCs (LCs)
Nonspecific esterase activity; complement receptors (CD11b, CD15b)	Intracellular BGs, Lag molecules, E-cadherin
Phagocytic properties	
CD68+ and express coagulation factor XIIIa	Lack CD68 and factor XIIIa expression
TNF, GM-CSF and IL-13 or IL-4 induce maturation	TGF- $\beta$ induces maturation

### FUNCTIONAL DIFFERENCES BETWEEN THE DIFFERENT DC SUBSETS

Although functional differences exist between the myeloid and lymphoid DCs, functional segregation within the myeloid DC lineage system also exists. Several in vitro studies have shown that CD14-derived DCs prime T cells to preferentially activate Th1 responses and IL-12 appears implicated in this process.<sup>34, 35</sup> CD14-derived DCs from CD34+ progenitors, but not CD1a-derived DCs, also activate naive B cells to secrete IgM in the presence of CD40L and IL-2.<sup>36</sup> In psoriasis and atopic asthma, distinct DC subtypes activate either Th1 or Th2 responses, respectively supporting the existence of pathophysiological associations between DC subtypes and Th cell subsets.<sup>37, 38</sup> This raises the possibility of redirecting tissue destructive T cell responses to nondamaging T cell responses in certain diseases. Additional observations indicate that CD14-derived DCs are increased in rheumatoid arthritis as it is predominantly associated with an inflammatory Th1 response.<sup>39-41</sup>

The thymic DCs expressing CD8a+ appear to be functionally different from CD8a- DCs in that they express Fas-L and can induce T cell apoptosis. Thymic DCs are also far less efficient at inducing T cell IL-2 cytokine production.<sup>18</sup> Thus, CD8a+ DCs may have regulatory properties, whereas CD8a- DCs seem to exert T cell stimulatory function. More functional studies have to be performed in order to fully evaluate the pro B lymphocyte DCs, and whether the pro B lymphocyte-derived DCs have unique functions that are not exhibited by the thymic DCs, remains to be elucidated.

It is clear from the forgoing discussion that DCs may develop from a myeloid or lymphoid lineage. The myeloid pathway of differentiation gives rise to DCs that home to peripheral tissues to take up and process exogenous Ags prior to migrating to the secondary lymphoid tissues to present Ags to naive T cells. Thymic DCs, on the other hand, perform a very different function being involved in the presentation of self-Ag to developing thymocytes and, hence, the subsequent deletion of autoreactive T cells. It would be appropriate for the precursors of thymic DCs to migrate to the thymus in an immature form and undergo development exposed only to self-Ag within the thymus.<sup>42</sup> Thus, the existence of alternative developmental pathways would be in keeping with the different functions of DCs in different tissues.

Further studies have revealed that CD8a+ DCs were at least equivalent to CD8a- DCs in stimulating both CD4 and CD8 responses in vivo and in vitro. A surprising recent finding is that, CD8a+ DCs, but not CD8a- DCs, produce significant levels of IL-12 and prime Th1 T cell response.<sup>43-46</sup> Thus, the immunoregulatory potential of DCs may depend less on ontology than on recent activatory or downregulatory stimuli. Appropriate induction of T cell tolerance or activation would be ensured by allowing DC behavior to be influenced by environmental signalling at the time of Ag encounter.

## MIGRATION AND FUNCTIONAL PROPERTIES OF DENDRITIC CELLS

Myeloid DCs are derived successively from proliferating progenitor cells and non proliferating precursors (especially monocytes). They migrate to and reside as immature DCs at body surfaces and interstitial spaces. Immature DCs have abundant MHC II products within intracellular compartments (MIICs) and respond rapidly to inflammatory cytokines and microbial products to produce mature T cell stimulatory DCs with abundant surface MHC II proteins (see Table 2 and 3); eventually leading to apoptotic death. Some researchers have reported that the Flt-3 ligand can mobilize DCs from proliferating progenitors in humans.<sup>47</sup> The immature DCs have many MIICs but require a maturation stimulus to irreversibly differentiate into active T cell stimulatory, mature DCs. Randolph (1998) has described an in vitro system involving monocytes reverse transmigration across an endothelial monolayer that offers a possible explanation.<sup>48</sup> This type of situation would occur when cells move from tissues to afferent lymph. It is possible that veiled DCs in lymph originate from monocytes in tissue that interact with the lymphoid endothelium to acquire the properties of immature DCs. If the monocytes also phagocytose particles before they reverse transmute, then the cells become typical mature DCs; the process occurs within 48 hours. The cells possess several markers (p55, DC-LAMP and CD83) that are expressed by mature DCs but are weak or absent in other leukocytes.<sup>49-51</sup> Dendritic cells that have matured from monocytes in this in vitro system also express very high levels of surface MHC class II and CD86 and, in complete contrast to monocytes, have lost CD14, CD32 and CD64, all within 48 hours of culture (see Tables 2 and 3).

**Table 2:** Characteristics of immature dendritic cells

### Characteristics of immature dendritic cells

- High intracellular MHC II in the form of MIICs
- Expression of CD1a
- Active endocytosis for certain particulates and proteins; presence of FcγR and active phagocytosis
- Deficient T cell sensitization in vitro
- Low/absent adhesive and costimulatory molecules (CD40/54/58/80/86)
- Low/absent CD25, CD83, p55, DEC-205, 2A1 antigen
- Responsive to GM-CSF, but not M-CSF and G-CSF
- Maturation inhibited by IL-10

**Table 3:** Characteristics of mature dendritic cells

### Characteristics of mature dendritic cells

- Cell shape: Numerous processes (veils, dendrites)
- Motility: Active process formation and movement
- Antigen capture: Macrophage mannose receptor, DEC-205 receptor
- Antigen presentation: High MHC class I and II expression
- Abundance of molecules for T cell binding and costimulation, (e.g. CD40, CD54/ICAM-1, CD58/LFA-3, CD80/B7-1 and CD86/B7-2)
- Cytokines: Abundant IL-12 production; resistance to IL-10 DC-restricted molecules: p55, CD83, S100b
- Absence of macrophage-restricted molecules and function: CD14, CD115/c-fms/M-CSF responsiveness, low CD68, myeloperoxidase and lysozyme, bulk endocytic activity (pinocytosis, phagocytosis)
- Stability: No reversion/conversion to macrophages/lymphocytes

Another source of DCs is the immature LC present within the epidermis. LCs are the prototype immature DC, as revealed by Schuler and Romani (1985 and 1989).<sup>52, 53</sup> Immature DCs lack or have low levels of several important accessory molecules that mediate binding and stimulation of T cells -CD40, 54, 58, 80 and 86.<sup>52, 53</sup> The MHC II molecules are primarily within the cell in MIICs that coexpress lysosomal-associated membrane proteins and HLA-DM or H-2M.<sup>54-56</sup> Randolph et al (1998) have reported that human LCs express high level of MDR-1, a multi-drug resistance receptor. LC migration in vitro from skin organ cultures is blocked by anti-bodies or drugs

(verapamil, reserpine) that block MDR-1 or P-glycoprotein.<sup>57</sup> There are at least 2 *mdr* genes, and one controls the extrusion of leukotrienes. Also, recent studies show that specific chemokines and chemokine receptors direct the movements of immature and mature DCs; in particular the inflammatory chemokines macrophage inflammatory protein (MIP)-1a and MIP-3a for mobilizing immature DCs and constitutive lymphoid chemokines (MIP-3b or EBI1-ligand chemokines (ELC), 6C-kine or secondary lymphoid tissue chemokines (SLC), for directing mature DCs to T cell areas in secondary lymphoid compartments.<sup>58-60</sup>

## MATURATION AND FUNCTION OF DENDRITIC CELLS

In most tissues, DCs are present in a so-called 'immature' state and are unable to stimulate T cells. Although these DCs lack the requisite accessory signals for T cell activation, such as CD40, CD54, CD80 and CD86, they are extremely well equipped to capture Ags in peripheral sites. Once they have acquired and processed the foreign Ags, they migrate to the T cell areas of lymph nodes (LNs) and the spleen, undergo maturation and stimulate an immune response.

Immature DCs have several features that allow them to capture Ag. Firstly, they can take up particles and microbes by phagocytosis.<sup>61, 62</sup> Secondly, they can form large pinocytotic vesicles in which extracellular fluid and solutes are sampled; a process called macropinocytosis.<sup>55</sup> And thirdly, they express receptors that mediate adsorptive endocytosis, including lectin receptors like the macrophage mannose receptor and DEC-205, as well as Fcγ and Fcε receptors.<sup>30,63</sup>

Macropinocytosis and receptor-mediated Ag uptake is very efficient, requiring picomolar and nanomolar concentration of Ag, much less than the micromolar levels typically required by other APCs. However, once DCs have captured Ags, which also provide the signal to mature, their ability to capture more Ag rapidly declines.

The captured Ags enter the endocytic pathway of the cell. In macrophages, most of the protein substrates are directed to the lysosomes, organelles with only a few MHC class II molecules, where the Ags are completely digested into amino acids. By contrast, DCs are able to produce large amounts of MHC class II-peptide complexes. This is due to the specialized, MHC class II rich compartments (MIICs) that are abundant in immature DCs.<sup>54, 64, 65</sup> During maturation of DCs, MIICs convert to non-lysosomal vesicles and discharge their MHC-peptide complexes on to the cell surface.<sup>65, 66</sup>

Once primed, the DCs migrate to secondary lymphoid compartments (e.g. LNs) to present Ag-peptide complexes to naïve CD4<sup>+</sup> T cells and CD8<sup>+</sup> cytotoxic T cells. Following education by Ag-loaded DCs in LNs, naïve CD4<sup>+</sup> T cells differentiate into memory helper T cells, which support the differentiation and expansion of CD8<sup>+</sup> CTLs and B cells. Helper T cells exert anti-tumour activity indirectly through the activation of important effector cells such as macrophages and CTLs, which are capable of eradicating tumour cells or virus-infected cells directly. DCs are able also to present Ags via the exogenous class I presentation pathway.<sup>67,68</sup> A dedicated peptide transporter translocates these peptides from the cytosol to the endoplasmic reticulum, where they bind to class I molecules. The peptide-bound MHC class I complexes migrate to the cell surface where they are displayed for T cells. This interaction generates CTLs, which have the capacity to eliminate virally infected cells and tumour cells.

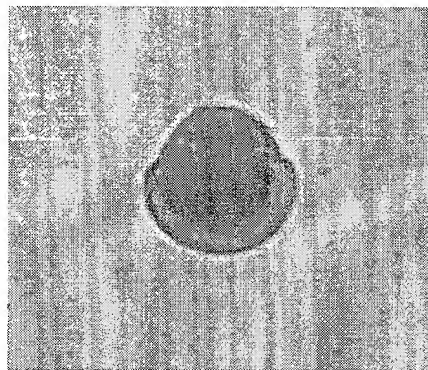
It is clear that the maturation of DCs is crucial for the initiation of immunity. This process is characterized by reduced Ag-capture capacity and increased surface expression of MHC and co-stimulatory molecules. However, the maturation of DCs is completed only upon interaction with T cells. It is characterized by loss of phagocytic capacity and expression of many other accessory molecules that interact with receptors on T cells to enhance adhesion and signalling (co-stimulation); for example, LFA-3/CD58, ICAM-1/CD54, B7-1/CD80, B7-2/CD86 and CD83.<sup>69,70</sup> (see Table 2 and 3) Expression of one or both of the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) on the DCs are essential for the effective activation of T lymphocytes, and, for IL-2 production.<sup>71</sup> These co-stimulatory molecules bind the CD28 molecules on T lymphocytes. If this fails to occur at the time of Ag

recognition by the TCR, an alternative T lymphocyte function may result, namely induction of anergy.<sup>54, 71</sup> Another CD80/86 ligand, CTLA-4, is also induced on activated T lymphocytes, and this may contribute a negative regulatory signal.<sup>72, 73</sup>

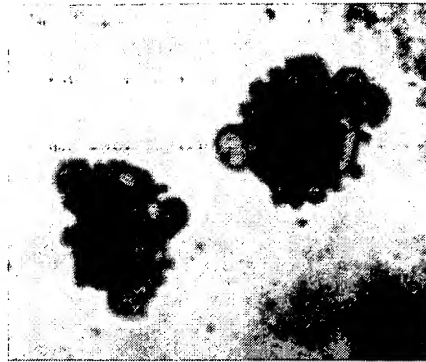
Dendritic cells are a major source of many cytokines, namely, interferon-alpha (IFN- $\alpha$ ), IL-1, IL-6, IL-7, IL-12 and IL-15 and also produce macrophage inflammatory protein (MIP1g), all of which are important in the elicitation of a primary immune response.<sup>74-80</sup> Also, there is evidence that the cytokine secretion pattern of the plastic-adherent monocyte-derived DCs (grown in GM-CSF and IL-4) can be induced along the Th1 (IL-12) or Th 2 (IL-10) cytokine secretory pathway. Interleukin-12 production is critical for the promotion of an effective cellular immune response by activating and differentiating T lymphocyte to the Th 1 pathway. Its secretion appears to be inhibited by various tumour-derived substances, including nitric oxide (NO), prostaglandin E2 (PGE2), IL-10, IFN- $\alpha$  itself, the p40 homodimer of IL-12, and transforming growth factor b (TGF-b), which is regarded predominantly as an immunosuppressive cytokine.<sup>74, 78-80</sup>

Dendritic cells, grown and matured in vitro, can synthesize IL-10 in a continuous manner. Zhou and Tedder (1995) have found IL-10 transcripts, in CD83+ cells isolated from peripheral blood by RT-PCR analysis, and de Saints-Vis et al. (1998) have observed IL-10 synthesis by CD14+ DCs of myeloid origin.<sup>74, 81</sup> It is well established that monocytes and macrophages synthesize IL-10.<sup>82</sup> It is also well known that IL-10 has an important regulatory role on monocyte function and on DC maturation.<sup>83-85</sup> Furthermore, IL-10 has been documented to have a significant inhibitory effect on several aspects of APC function, for example, the expression of co-stimulatory molecules and the ability to synthesize IL-12.<sup>83, 85, 86</sup> Importantly, IL-10 treated-DCs can be tolerogenic.<sup>87-90</sup> Dendritic cells secreting IL-10 exhibit minimal or no stimulatory properties in primary MLRs and are markedly inhibitory to T cell proliferation induced by polyclonal activators.<sup>91</sup> Thus, IL-10 producing DCs are functionally and phenotypically inhibitory accessory cells and putatively tolerogenic.<sup>92</sup>

**Figure 1:** Morphological characteristic of human immature DC from peripheral blood after immuno-magnetic bead isolation (Wright-Giemsa, x 600)



**Figure 2:** Morphological characteristic of human mature and activated DC from lymph node after immuno-magnetic bead isolation (Wright-Giemsa, x 600)



### DC DEATH (APOPTOSIS)

Although previous studies have demonstrated that developmentally and functionally end-stage DCs undergo apoptotic cell death, the possibility that apoptosis contributes to the regulation of the DC pathway at others stages of DC development has not been extensively explored.<sup>93</sup>

Functionally distinct apoptotic schedules were associated with different phases of DC development when multipotent CD34+ progenitor cells were treated with GM-CSF, TNF ± SCF (c-kit ligand).<sup>94</sup> During early phases of growth (days 0-3), unselected progenitors underwent apoptosis. During intermediate stages (days 3-7), high levels of apoptosis resulted in the preferential selection of DC precursors, as revealed by the substantial expansion of DR+ CD33+ CD13+ cells. Late apoptosis (after day 10) was associated with the death of mature DCs. Apoptotic events surrounding the earlier periods were related to the exogenous addition of TNF-α and appeared to be mediated by fas. In contrast, those events associated with terminally differentiated DCs were fas-independent, because there was no correlation between fas expression and cell death. Recent studies by Canque et al (1998) have shown that the inclusion of TNF-α during DC development produces apoptotic events that selectively promote the CD1a-dependent DC pathway from GM-CSF, TNF-α treated CD34+ cord blood progenitors cells, lending support to the above observations.<sup>95</sup> The susceptibility to apoptosis was remarkably decreased when DC precursors were treated with GM-CSF or IL-3, further supporting that these cytokines are viability (anti-death) factors for DCs.<sup>95</sup>

A number of researchers have concentrated on TRANCE or RANK-1, a TNF member identified by several groups recently. It increases the viability of mature myeloid DCs; both mouse DCs generated from the marrow or human DCs developed from monocytes.<sup>96, 97</sup> TRANCE does not alter adhesion and co-stimulatory molecules like ICAM and B7, but it does make the mature DCs stay alive longer and express several cytokines genes, including IL-1, IL-6, IL-12 and IL-15. These responses are important features of mature DCs, which are sometimes referred to as activated and superactivated. When the mature DCs encounters the correct TNF family member, its viability is improved and cytokine production is enhanced, thus, creating a longer lasting and more effective Ag presenting cell.<sup>96, 97</sup>

These studies show that, at least within the myeloid lineage, the activation of distinct apoptotic processes regulates DC development and homeostasis. Although suppression of apoptosis may prolong the survival of mature DCs, activation of apoptosis is required for the selective expansion of multipotent DC progenitors. These data also provide insight into the mechanisms of myeloid lineage selection by cytokines such as TNF-α, which may promote both cell death and survival.

### DENDRITIC CELL DISTRIBUTION

#### Dendritic cells in peripheral blood

Although human PBDCs were first isolated in 1982 their phenotype has been poorly defined due to their low numbers and the lack of specific markers by which they could be clearly identified. Hitherto, purification of PBDCs

has relied upon either sequential depletion of other PBMC subsets or on their physical properties, such as their capacity for transient adherence to plastic and their low density, thereby, permitting separation over density gradients.<sup>98</sup> Using such techniques, it has been demonstrated that the most potent allostimulatory fraction of PBMCs possessed a phenotype characterized by high levels of HLA class II expression and absence of markers for other cell lineages.<sup>99</sup> These studies and new techniques, such as immuno-magnetic bead depletion and fluorescence flow cytometry, have enabled the further characterization of PBDCs and the demonstration of at least two subpopulations of cells.<sup>100-102</sup>

Using three-colour flow cytometry, two subpopulations of HLA-DR+ PBDCs, characterized by the phenotypes CD2-CD13- CD33dim CD11c- HLA-DR+ and CD2+ CD13+ CD33bright CD11c+ HLA-DR+ respectively, have been documented.<sup>100, 101, 103</sup> The morphology of these two subtypes differ, the CD11c- population possess a lymphoid appearance and the CD11c+ population possess a monocytoïd morphology.<sup>101, 103</sup> Further, both subsets lack expression of the LC marker CD1a and express only low levels of adhesion and co-stimulation molecules CD80, CD86 and CD40, suggesting that these cells are relatively immature.<sup>100, 101, 103</sup> However, when cultured, both populations develop into cells with typical DC morphology that express high levels of adhesion and co-stimulatory molecules and possess potent allostimulatory function.<sup>100,101</sup> The CD33dim subset possess a lower allostimulatory activity which increases in culture along with expression of both HLA-DR and CD33.<sup>100</sup> However, it has recently been demonstrated that although both subsets are stimulatory in the MLR only the CD2+ subset is capable of presenting Ag to naïve CD4+ T cells suggesting that these subsets of DCs may be functionally distinct.<sup>104</sup>

In order to facilitate the study of PBDCs, two markers specific for these cells have been identified in recent years. CD83 is a 45 kd member of the immunoglobulin superfamily that is virtually specific for DCs derived from the peripheral blood.<sup>49,105</sup> Although CD83 was not originally found on freshly isolated DCs but only after in vitro culture,<sup>49</sup> a later report identified a small subset of DCs that expressed CD83, when freshly isolated, and a larger subset that upregulated CD83 expression upon culture.<sup>102</sup> The 55 kd actin bundling protein (p55) is a highly conserved protein important in the rearrangement of the cytoskeleton, cell motility and phagocytosis. Monoclonal antibodies against p55 detect this protein in 96% of PBDCs but not in other PBMC populations and, therefore, may be useful in the quantification and purification of PBDCs.<sup>50</sup>

While DC subsets have been defined in the peripheral blood their characterization has remained inconsistent reflecting, in part, the different purification protocols and MAbS used to define each subset. It remains to be established if one subset of PBDCs is the precursor for the other or whether each subset has developed along a distinct maturational/functional pathway. It has also been suggested that one subset may be more mature by virtue of being tissue-derived and migrating to lymph nodes or spleen whilst the less mature is directly derived from the bone marrow.<sup>101</sup>

### **Dendritic cells in peripheral tissues and lymph nodes**

Dendritic cells have been identified within the interstitial space of most human tissues although notable exceptions are the absence of DCs in the cornea and central nervous system.<sup>106, 107</sup> Within tissues, DCs exist as trace populations and may be identified by the combination of DC morphology and immunohistochemical labeling to demonstrate the expression of high levels of HLA-DR, CD1a (on LCs in the epidermis) and S100, and the absence of other lineage markers.<sup>108, 109</sup> There is evidence that tissue DCs are derived from circulating blood precursors which bind to the endothelial receptors ICAM-1, V-CAM-1 and E-selectin through the expression of CD11a/CD18 and CD49d and cutaneous lymphocyte antigen (CLA), respectively.<sup>110, 111</sup> This recruitment of DCs to tissue may be partly mediated by the local production of cytokines such as GM-CSF and by systemic signals such as bacterial lipopolysaccharide (LPS).<sup>112, 113</sup> Within tissues, DCs reside in an intermediate stage of maturity as cells specialized for Ag uptake and processing. Tissue DCs take up Ag both in the fluid phase by macropinocytosis and

via receptor-mediated endocytosis using the mannose receptor to ingest glycosylated Ag, and via the FcγRII (CD32) cell surface receptors take up antibody-bound Ag.<sup>114</sup> Endocytosed particulate matter is channeled via an acidic vacuolar route to the intracellular class II compartment where antigenic peptides are assembled onto MHC class II molecules for presentation to T cells.<sup>114</sup> The functional status of the DCs is regulated by a variety of cytokines and upon exposure to TNF-α, IL-1 and bacterial LPS, DCs undergo further maturation and migration.<sup>115-117</sup> This involves downregulation of Ag uptake and processing, increased expression of the co-stimulatory molecules CD40, CD54, CD80 and CD86, enhanced Ag presenting function<sup>30, 118</sup> and migration from the tissues to the lymph nodes and spleen.<sup>119-121</sup> These changes in DC maturity and function, following exposure to cytokines and products of bacterial and cellular degradation, conform to the 'danger' hypothesis for activation of the immune response, as proposed by Marzinger (1994).<sup>122</sup>

DCs migrate to the secondary lymphoid tissues via the afferent lymphatics as veiled cells, so called because of their characteristic sheet-like lamellipodia. Cannulation of dermal afferent lymphatic vessels in human subjects has demonstrated an increase in CD1a+ DCs leaving the skin following exposure to contact sensitizers.<sup>123</sup> There is also evidence from animal transplantation models that solid tissue DCs may migrate via the blood to the spleen.<sup>121</sup> The mechanisms of homing to the LNs and spleen are not fully understood but recent evidence suggests that expression of certain isoforms of the hyaluronic acid receptor (CD44) may be important.<sup>124</sup> In LNs, DCs reside within the T cell paracortical regions as interdigitating DCs (IDCs), whilst in the spleen they are located in the marginal zones at the periphery of the periarterial sheaths.<sup>106</sup> The IDCs nonspecifically cluster T cells through their expression of adhesion molecules and present Ag in association with class II molecules. Antigen-specific T cells may then proliferate provided those co-stimulatory signals are communicated via CD40, CD80 and CD86 to their ligands on T cells. Production of cytokines such as IL-12 by the DCs further directs the evolving immune response along a Th1 pathway.<sup>75</sup> It is apparent that the responding lymphocytes signal back to the DCs via MHC-TCR and CD40-CD40L interactions to promote further upregulation of DC co-stimulatory function.<sup>76</sup> In addition to the IDCs of the T cell regions, DCs that are distinct from follicular DCs have recently been described in the B cell germinal centre.<sup>77</sup> This suggests that they may play a role in T-dependent B cell memory immune responses. Kinetic studies in mice demonstrate a rapid DC turnover and life cycle in LNs, with DCs undergoing apoptosis after presentation of Ags to T cells.<sup>106, 125</sup>

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